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Regulation of the HCMV UL98 Promoter by Cooperation of the Cyclic AMP Response Element and Gamma Interferon Response Element

Tabmitha Patricia Young Jervey
Old Dominion University

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**REGULATION OF THE HCMV UL98 PROMOTER BY
COOPERATION OF THE CYCLIC AMP RESPONSE ELEMENT
AND GAMMA INTERFERON RESPONSE ELEMENT**

by

**Tabmitha Patricia Young Jervey
B.S. May 1991, Norfolk State University, Norfolk, Virginia**

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Approved by:

Ann E. Campbell (Director)

Kenneth Somers (Member)

Timothy J. Bos (Member)

Julie A. Kerry (Member)

ABSTRACT

REGULATION OF THE HCMV UL98 PROMOTER BY COOPERATION OF THE CYCLIC AMP RESPONSE ELEMENT AND GAMMA INTERFERON RESPONSE ELEMENT

Tabmitha Patricia Young Jervey
Eastern Virginia Medical School and Old Dominion University, 2001
Director: Dr. Richard M. Stenberg

The HCMV UL98 early alkaline exonuclease gene promoter was examined to determine the DNA sequences as well as viral and/or cellular proteins functional in the regulation of this early gene. To assess promoter activation, UL98 promoter sequences were first cloned upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and cotransfected with expression plasmids which express the HCMV major immediate early (IE) proteins IE72 and IE86. To more specifically determine the importance of individual cis-acting elements in UL98 promoter activation, the promoter region underwent mutagenesis to delete or alter sequences. The variant promoters were again cloned into a reporter-CAT construct and analyzed in transient transfection assays to assess changes in promoter activity in response to viral or virally induced proteins.

Analysis of promoter activation indicated that the UL98 promoter required the presence of the IE72 and IE86 immediate early (IE) proteins. In comparison to a prototypical early promoter which regulates the polymerase (pol) gene, UL98 promoter activation levels were equal to or even greater than that of pol in response to the IE transactivators. In the presence of all viral proteins, activation of the UL98 promoter continually increased when analyzed at 24, 48, and 72 hours.

Deletion analysis showed that a 13 bp sequence located between -64 and -51 is

required for UL98 promoter activation by IE proteins. Site-directed mutations generated in two cellular transcription factor binding sites resulted in a drastic reduction in promoter activation. A mutation in the cyclic AMP response element (CRE) (-82 to -75) resulted in a 70% loss of promoter activation. The UL98 promoter was also poorly activated in the presence of a mutation generated in the gamma interferon response element (γ IRE) (-37 to -30), a transcription factor binding site downstream of -51. Binding and competition experiments via gel mobility shift assays provided conclusive evidence that the CREB protein binds the CRE site in the UL98 promoter.

These data indicate that the UL98 early promoter is regulated primarily by the CRE and gamma IRE sequences. UL98 promoter activation therefore relies upon the presence of a combination of elements in their defined flanking positions.

This thesis is dedicated to Jesus Christ my Lord and Savior, to my devoted and loving husband Bryan, my children, my father, my mother whose love lifted me, and my siblings.

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TABLE OF CONTENTS

	Page
LIST OF FIGURES	ix
 CHAPTER	
I. INTRODUCTION.....	1
Cytomegaloviruses: classification and general characteristics	1
Human cytomegalovirus: discovery and isolation.....	2
Virion Structure	3
Organization of the viral genome	4
Pathogenesis of HCMV Disease	6
Antiviral therapies for treatment and prophylaxis of HCMV	14
Vaccines	16
The virus growth cycle.....	17
Herpesvirus Alkaline Nuclease	33
 II. SPECIFIC OBJECTIVES	36
 III. MATERIALS AND METHODS	39
Cells	39
Virus.....	39
IE gene region plasmids and cDNAs	39
<i>Xba</i> I-C (pCBX8.1) plasmid.....	40
pUL98CAT	40
pdH309CAT	40
Deletion Mutants	41
Substitution Mutations	42
Sequencing of plasmid DNAs	43
Transient transfection and superinfection assays	44
Chloramphenicol Acetyltransferase (CAT) Assay	45
Preparation of Nuclear Extracts	46
CREB protein	47
Gel Mobility Shift Analysis	47
Radiolabeling of DNA fragments or ds synthetic oligonucleotides	47
Cloning Methodology	50
 IV. RESULTS.....	56
Identification of the UL98 promoter relative to the putative TATA box and the CAP site at +1: cloning and characterization of the UL98 promoter in a CAT-reporter construct	56
Deletion analysis of the UL98 promoter.....	62

Mutational analysis of specific response elements in the UL98 promoter	76
Analysis of protein-DNA interaction in UL98 promoter activation.....	90
V. DISCUSSION.....	113
Activation of the HCMV UL98 Early Promoter.....	113
Deletion Analysis of the UL98 Promoter	117
Analysis by Substitution Mutation (CRE and TCF-1)	120
Analysis by Substitution Mutation (SIE and γ IRE).....	127
Significance of These Studies and Future Directions.....	133
VI. CONCLUSIONS	138
REFERENCES	140
APPENDIXES	
I. List and description of plasmid DNAs obtained from other sources or previously constructed	173
II. List of plasmids constructed during this study	174
III. List of primers used for the generation of mutation by overlapping PCR mutagenesis.....	177
VITA	179

LIST OF FIGURES

Figure	Page
1. Sequence of the putative UL98 promoter	57
2. Generation of the pUL98CAT construct.	58
3. Activation of early promoters ppolCAT and pUL98CAT by IE1 (IE72) and IE2 (IE86) gene products	59
4. Activation of pUL98CAT by IE1 and/ or IE2	61
5. Removal of the 3' <i>Hind</i> III site to generate pdH309CAT	63
6. Construction of the UL98 deletion mutants utilizing pdH309CAT	65
7. A schematic representation of site-directed mutagenesis by overlapping PCR	66
8. Insertion of the <i>Hind</i> III site for the construction of deletions in the UL98 promoter	69
9. UL98 promoter deletion mutants	71
10. Responsiveness of promoter deletion mutants to IE proteins	72
11. UL98 promoter deletion mutants activated by HCMV infection	73
12. Model of UL98 promoter activation	75
13. A schematic representation of the UL98 promoter mutants	77
14. Activation of site-directed promoter mutants in the presence of IE proteins	78
15. Time course of activation of pUL98CAT and mutants (CRE/TCF element) following superinfection with HCMV	80
16. Time course of activation of pUL98CAT and mutants (SIE/ γ IRE) following superinfection with HCMV	81
17. CRE/TCF element mutations in the context of the -85 truncated promoter following superinfection with HCMV	84

Figure	Page
18. SIE/ γ IRE mutations in the context of the -85 truncated promoter following superinfection with HCMV.....	85
19. Assessment of mutated elements in the context of the -64 truncated promoter by transfection/superinfection assay.....	87
20. SIE and γ IRE mutations in the context of the -51 truncated promoter.....	89
21. Binding of infected cell proteins to a UL98 promoter fragment.....	91
22. Competition analysis of the UL98 promoter fragment.....	93
23. CRE/TCF element probes and competitors generated for gel mobility shift assays.....	94
24. Binding and competition of the UL98 CRE sequence.....	96
25. Mutant CRE probe (CREm) in competition assays.....	97
26. Identification of protein binding the UL98 promoter.....	99
27. Confirmation of cyclic AMP response element as a binding protein of the UL98 promoter.....	100
28. Binding and competition of the UL98 TCF sequence.....	102
29. SIE/ γ IRE probes and competitors generated for gel mobility shift assays.....	103
30. Binding and competition of the UL98 SIE and γ IRE sequences.....	105
31. Competition analysis by gel mobility shift assay utilizing mutant probes....	106
32. Competition analysis by gel mobility shift assay utilizing double and triple mutant probes.....	108
33. Supershift assays utilizing IRF-3 antibody.....	109
34. Confirmation of specific IRF-3 binding.....	111

CHAPTER I

INTRODUCTION

Cytomegaloviruses: classification and general characteristics

Human cytomegalovirus (HCMV) is a large animal DNA virus categorized under the family *Herpesviridae*, subfamily *betaherpesvirinae*, and the genera *cytomegaloviruses*. Eight human herpesviruses (HHV) have been identified to date. HCMV (HHV5) is the fifth of the herpesviruses which includes herpes simplex type 1 (HSV 1), herpes simplex type 2 (HSV 2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV6), human herpesvirus 7 (HHV7), and Kaposi's-sarcoma associated human herpesvirus 8 (HHV 8) (153). As a betaherpesvirus member, cytomegaloviruses are characterized by the ability to persist in a restricted host range. Previously known as salivary gland viruses, the virus-induced cytopathic effects called cytomegalia and the viral role in congenitally acquired cytomegalic inclusion disease (CID) prompted the new name cytomegalovirus (reviewed in reference 125).

In addition to the unique characteristic of salivary gland tropism, HCMV also grows slowly in culture and is species specific. It is highly distributed in nature with greater than 50% of the U.S. population outside urban centers acquiring HCMV in early childhood. Greater than 90% of the U.S. population in urban centers are infected with HCMV. HCMV establishes a persistent infection which can lie latent in the host for life causing no harm. However, the pathogenic effects of HCMV have been more apparent in recent years due to its association with patients having suppressed immune systems as a

The model for this thesis is Journal of Virology.

result of acquired immune deficiency syndrome (AIDS) as well as transplant patient recipients under post immunosuppressive therapies.

Human cytomegalovirus: Discovery and isolation.

Cytomegalovirus infection characteristically results in enlarged cells with intranuclear inclusions very similar to those produced by other herpesviruses (i.e., HSV, VZV) (12). In the early 1900s, the cellular changes observed in patient tissues were thought to have been a consequence of protozoan or syphilitic infection. Jesionek and Kiolemenoglou observed the presence of protozoan-like cells in several organs of a leptic fetus (15, 70). Ribbert reported observing similar cells on two separate occasions (15, 70). Infant organs such as the submaxillary glands were noted postmortem as having similar cellular changes (70). It was from these observations that the term “cytomegalic inclusion disease” (CID) was named. In 1921, Lipschutz compared the intranuclear inclusions he observed to herpetic lesions thereby proposing that a virus may be the causative agent for CID (15, 70). Cole and Kuttner provided the initial experimental evidence for virus induced CID (30). Using a guinea pig model, they showed that filtered homogenates of salivary glands with intranuclear inclusions were indeed infectious to animals. The animals had no prior evidence of CID. A causative agent was proposed soon after salivary gland tissues from infected animals were compared to the infected salivary glands from human tissue. The virus was named the “salivary gland virus”.

In the 1950's, the virus was isolated by three independent laboratories. Smith and Weller, in 1956 and 1957, respectively, each isolated the virus from infants with CID (175, 220). The term “cytomegalovirus” was first used by Weller et al. to reflect the

cytopathology produced by the virus (219). Rowe isolated the laboratory adapted strain of the virus now called AD169 (155). The adenoidal tissue was taken from a child who had undergone tonsil and adenoidectomies. Instead of isolating adenovirus, the AD169 strain of the virus was fortuitously isolated. Cytomegalovirus isolated from humans finally became known as human cytomegalovirus (HCMV). Serology indicated that HCMV infection was a common, usually subclinical infection that occurs worldwide. In infants, the elderly, and especially the immunocompromised, the infection becomes more severe. CMV classification as a member of the *Herpesviridae* family was decided based on viral morphology and its DNA composition.

Virion Structure

The structure and composition of the CMV virion resembles that of other virions in the herpesvirus family. The virion of cytomegalovirus is the most extensively characterized of the beta herpesvirus group (50). Pleomorphic in shape, mature virions usually range in size from 150 to 200 nm. The components include a large double-stranded DNA, a central core, an icosahedral capsid, a tegument, and the trilaminate envelope. The genome is linear and is located within the central core of the particle. The capsid, composed of 162 capsomeres, surrounds the core with a diameter of approximately 110 nanometers (nm). At least four capsid proteins are present in virions. 90% of the capsid protein consists of the major capsid protein (MCP, UL86) (153.8 kD) (51), the minor capsid protein (mCP, UL85) of 34.6 kD, the minor capsid binding protein (mC-BP, UL46) of 33 kD, and the smallest capsid protein (scp, UL48/49) of 8.5 kD. The tegument, which lies between the capsid and the envelope, has not yet been characterized

functionally. The tegument contains twenty proteins on its surface and makes up approximately 40 % of the CMV virion protein mass. Five predominant proteins of the tegument include UL48 (250 kD), UL47 or hmw-BP (110 kD), UL32 or pp150 (113 kD), UL82 or pp71 (62 kD), and the UL83 or pp65 (63 kD). The latter three proteins are phosphorylated *in vivo* and *in vitro* (74, 112, 151) and only the pp71 phosphoprotein has been functionally characterized; a transcriptional transactivator of the HCMV major immediate early promoter (124). Lastly, the virion envelope, which surrounds the tegument, is derived from the infected cell plasma membrane and contains most of the virion glycoproteins. Of the eight identified glycoproteins in CMV, only four have been confirmed as HSV homologs: gB, gH, gL, and gM (16). While other glycoproteins have been identified, their function has not yet been elucidated. gB studies suggest that this glycoprotein participates in the attachment and fusion of the virion to the host cell membrane by binding heparin, a widely expressed cell surface protein (reviewed in 153). gL has been shown to associate with the gH glycoprotein and function in the fusion process. gM is thought to participate in the heparin-binding activity due to its presence in the gCII complex (83).

Organization of the viral genome

Characterization of the herpesvirus DNA to date is based on size, conformation, base composition, and sequence arrangement. Herpesvirus DNA was found to be linear, double-stranded, and the molecular weight ranges from 120 to 230 kilobase pairs (kbp) (153). Base composition of the different herpesvirus DNAs range from 31 to 75 G+C mole % (153). The sequence arrangement of the DNA's among the herpesviruses is a

most interesting feature. Reiterations of terminal repeats greater than 100 bp occur both within the genome and/or at the termini. This characteristic allows for the classification of the genome varieties into six different groups from A to F depending on how the repeats are displayed (153). Group A is represented by the catfish herpesvirus in which the direct repeats are present at the termini. The herpes samiri virus comprises group B where terminal sequences are directly repeated several times at both ends. In group C, the direct terminal repeats are smaller than 100 bp (Epstein Barr Virus). The varicella zoster virus represents group D where terminal sequences at one terminus is repeated internally in the opposite orientation. In group E, sequences from both termini are repeated in an inverted orientation and are juxtaposed internally dividing the genome into two components. Each consists of unique sequences flanked by inverted repeats. Herpes simplex virus and the human cytomegalovirus represent group E. Lastly, group F contains sequences at two termini not identical. This group, represented by the tupaia herpesvirus, is neither repeated directly nor inversely (reviewed in 153).

The HCMV genome has been classified as a class E genome because the reiterated sequences occur at the termini and within the genome in the opposite orientation separating two different sequences into unique long (UL) and unique short (US) domains. The two domains can be rearranged or inverted to produce four different isomers of the HCMV genome. The genome of HCMV (AD169 strain) has been sequenced in total (24) and contains 208 predicted open reading frames (ORFs). The genome is divided into seven conserved sequence blocks (A to G) arranged in the order ABCDEFG (125). Although other herpesviruses contain sequence blocks, the arrangement or order is slightly different. The products from the conserved blocks

appear to function in DNA replication, DNA repair, nucleotide metabolism, or virion structure (125).

Clinical isolates as well as laboratory-adapted strains exist for HCMV. Previous studies show that differences in virulence among the various strains is dependent upon factors such as the inclusive genome sequences, cell tropism, and the presence of specific glycoproteins. Clinical isolates from patients as well as the low-passage Toledo strain contain greater than 13 kbp more of DNA than the high-passage AD169 and Towne strain (21). The 13 kbp region codes for putative glycoproteins. It has been observed in studies on cell tropism that clinical HCMV isolates have increased growth properties in endothelial cells compared with growth in fibroblasts (64,211). In experiments which detect infectious foci, clinical isolates were able to replicate well as exhibited by spread in endothelial cells, but AD169 and Towne strains did not induce infectious foci at all (172).

Pathogenesis of HCMV Disease

HCMV exists as a ubiquitous pathogen and a majority of the human population is serologically positive by young adulthood. Asymptomatic lifelong persistence is one of the striking characteristics of HCMV in an immunocompetent host. An unresolved issue regarding the state of HCMV in the normal individual is whether the virus produces low levels of infectious virus which is considered a chronic state, or if the virus truly lies latent producing no detectable infectious virus (15,19, 171). Viral persistence is of great concern since reactivation of HCMV in immunocompromised patients is an increasing problem.

HCMV persistence has been proposed to occur in macrophages and endothelial cells (reviewed in 42). Monocytes/ macrophages and endothelial cells are key participants in latent and acute infection from HCMV. In seropositive individuals, the source of virus is peripheral blood mononuclear cells (PBMCs). HCMV is transmitted to patients through the transfusion of the leukocyte fraction of peripheral blood. In patients with acute disease, HCMV is isolated from mononuclear and polymorphonuclear cell fractions. Separation of the PBMC population has resulted in the identification of monocytes as the predominant HCMV infected cell type (42,171). Ordinarily, HCMV natural infection of the PBMCs is infrequent and it is generally rare to find a productive infection in peripheral blood cells such as monocytes. When infection is observed in monocytes, however, it is low in frequency and viral expression is limited to immediate early events (171). Monocytes, therefore, are thought to both carry and disseminate virus evading the immune system by restricted gene expression. The differentiation of monocytes into macrophages may influence the ability of virus to replicate in these cells (177). In macrophages, HCMV infection is evident by the detection of virus and late viral gene expression (170). Epithelial, endothelial, and neuronal cells are productively infected with HCMV during an active infection. Vascular endothelial cells are another potential site for HCMV persistence (42). In vitro infection of monocyte derived macrophages can transmit virus to cultured endothelial cells via haematogenous seeding of endothelial cells. In particular, microvascular endothelial cells form the interface between peripheral blood and the underlying tissue. The interactions between these cell types may mediate the HCMV persistence as well as viral dissemination.

An HCMV primary infection is commonly initiated during childhood first infecting the ductal epithelial cells of the salivary gland. Transmission frequently transpires via person to person contact (e.g. urine, saliva). Virus is present and likely shed from the bodily secretions of a seropositive adult through tears, semen, cervical and vaginal excretions, breastmilk, blood, feces, and oropharyngeal secretions (reviewed in 15). In a healthy host, a clinical manifestation of the virus is a rare occurrence (reviewed in reference 125). Due to the *in vitro* properties of the virus, such as restricted cell tropism, limited cell to cell spread, and slow replication, pathogenicity often goes undetected. This also provides evidence for efficient immunity within the host. Symptoms characteristic of a common cold have been documented in subclinical cases. More severe cases, less common though, include a syndrome much like mononucleosis although not EBV derived. Fever, myalgia, pneumonia, hepatitis, and aseptic meningitis have also been recorded as occurring as a result of a primary infection or reactivation in a seropositive healthy individual.

As mentioned previously, the virus cohabitates with a seropositive immunocompetent host for life and it is possible that no virus is ever detected. However, in the case of individuals who are immunosuppressed, a primary infection as well as reactivation of HCMV can occur resulting in a severe clinical outcome. A HCMV infection/ reactivation either in a newborn not yet immunocompetent, or immunosuppression in transplant recipients, cancer patients, and AIDS patients may lead to cases of severe HCMV infection (reviewed in reference 15). In the case of fetal HCMV infections, the virus is acquired through crossing the placental barrier. This infection is considered one of the most prevalent congenital viral infection in humans.

Approximately 40,000 of newborns per year in the United States are infected and a significant percentage of the infants have long-term neurological damage (15). The seriousness of the viral infection is largely dependent upon the viral inoculum supplied by the mother, the virulence of HCMV, and the gestational age at the time of viral infection. HCMV intrauterine infection causes neurological damage to the fetus and thus accounts for the majority of the morbidity observed (15). While hearing loss is the most common neurologic abnormality, mental retardation and chorioretinitis have also been documented. Progressive hearing loss in the early years of life is likely evident of an ongoing CNS infection. Other symptoms occur as well, which include hepatosplenomegaly, thrombocytopenia, microcephaly, chorioretinitis, and hepatitis (15).

In allograft recipients, HCMV is a significant pathogen and contributes to disease not only in the transplantation process, but also in the post-transplantation period. Greater than 60% of solid organ allografts, cardiac and hepatic in particular, develop HCMV infection (40, 41). This may originate from the transplanted organ (54). As previously mentioned, blood products from transfused blood are an additional source of infection. Seropositive donor to seronegative recipient is the most common cause of primary infection resulting in severe HCMV infection with obvious HCMV disease. In the post-transplantation period, the level of immunosuppression influences the outcome of HCMV infections. Therapies directed at the elimination of T-lymphocytes, namely antithymocyte globulin and monoclonal antibodies directed at CD3+ lymphocytes in renal transplant recipients, have been associated with more frequent and severe HCMV infection (15,26,156).

The lack of survival of the transplanted graft has been associated with HCMV infection; in particular, a decreased graft survival in renal transplantation due to glomerulopathy (31). Cardiac graft recipients often develop coronary artery atherosclerosis linked to infection by human CMV (183). In bone marrow recipients, a complex relationship is thought to exist between HCMV infection and graft versus host disease (GVHD). This theory, however, remains debatable. The incidence of HCMV infection post bone marrow transplant ranges from 32% to 70% (15).

HCMV is the most important opportunistic infection encountered in patients with AIDS. The invasiveness of an HCMV infection is inversely proportional to the peripheral CD4+ lymphocyte count (130, 143). HCMV infection in the lungs, central nervous system (CNS) (reviewed in reference 222), and gastrointestinal tract (GI) are the three main organs most frequently reported. Pneumonitis is one of several life-threatening infections in AIDS patients. CMV retinitis is the primary manifestation of a CNS infection and the potential for severe loss of sight is likely if left untreated. Encephalitis has also been described after a CMV CNS infection. When localized in the epithelial tissue of the GI tract, HCMV has been implicated as the etiologic agent in the wasting syndrome characterized by rapid weight loss as well as excessive vomiting (98).

The fact that HCMV is widely disseminated is well documented, with HCMV being present in virtually all organs of the infected host (reviewed in reference 15). Monocytes appear to be the primary carrier of HCMV in the host. Virus replication is targeted to epithelial, endothelial, and glial cells (125). Microscopically, the cells appear large with decreased cytoplasm and a large nuclei. The nuclei, in most instances, show

marginated chromatin and notable intranuclear inclusions surrounded by a clear halo.

The numerous inclusions are often referred to as “owl’s eyes” (15).

The role of monocytes and macrophages in CMV infection have been largely characterized in the murine model of CMV. Stoddart et al. showed that murine cytomegalovirus (MCMV) infected monocytes may be functional in disseminating the virus to the spleen, liver, and lung (197). Macrophages have been implicated as being the site of productive viral replication and in the latency of MCMV (122, 146). Macrophages infected with CMV are also known to produce cytokines with antiviral activity. These include the tumor necrosis factor alpha (TNF α), interleukins (IL)-1 β , -6, -8, -12, and interferon (IFN)- α/β (reviewed in reference 19, 125, 137, 157). The role of macrophages in the production of IFN α/β provides direct antiviral activity against CMV in the early stages of infection, however this does not result in virus elimination. Therefore, while the production of cytokines via macrophages is advantageous for the host, the role of the macrophage in harboring infectious virus for spread gives this cell type a dual role. Which of the dual macrophage roles win out in the outcome of infection is dependent upon multiple factors.

One important factor in the role of macrophages in response to viral infection is interferon. Generally, interferons (IFNs) are cytokines that render cells resistant to viral infection, regulate cell growth, and differentiation (reviewed in reference 207). IFNs are divided into two classes based on their ability to bind distinct receptors: Type I or IFN alpha/ beta (α/β) and type II or IFN gamma (γ). IFNs also induce distinct but overlapping sets of genes in target cells via activation of latent cytoplasmic factors by phosphorylation rendering them active and functional within the nucleus. IFN α

stimulates signal transducers and activators of transcription (STAT) phosphorylation in the cytoplasm. The phosphorylated STAT proteins are then translocated into the nucleus where they form an interferon stimulated gene factor (ISGF)3 complex and bind the DNA sequence interferon stimulated response element (ISRE). IFN γ , on the other hand, stimulates a different cytoplasmic complex initiating phosphorylation and translocation of the gamma interferon activation factor (GAF) complex to the nucleus. GAF then binds the DNA sequence referred to as gamma interferon activation site (GAS). Whether stimulated via type I or type II IFN, the result is induction of genes that initiate an antiviral state in response to viral infection.

Although the activation of cellular genes via IFNs function in antiviral defense, studies show that human viruses can inhibit this step. In an adenovirus infection, the E1A gene product can interfere with the DNA-binding of interferon stimulated gene factor (ISGF)3 (58). Therefore, the transcriptional suppression of cellular interferon stimulated genes (ISG) results. The infection of cells with mutant E1A deficient adenovirus can cause the activation of ISRE-containing genes. HCMV infection can complement E1A mutants, however is unable to alter IFN α/β regulated ISGs (131). Instead, HCMV infection increases the activation of ISRE controlled ISG54 gene without de novo protein synthesis. In this study, a novel transcription complex is induced in response to HCMV infection (131). An interferon regulatory factor (IRF)-3 in complex with CREB binding protein (CBP) is called the cytomegalovirus-induced interferon-stimulated response element binding factor (CIF) is formed in response to HCMV infection.

IFN- γ , plays a role in the differentiation of monocytes to macrophages. This cytokine component, in addition to TNF α , was shown to render macrophages fully permissive for HCMV replication (177). It appears that HCMV takes advantage of two major cytokines with antiviral activity to obtain a specific state of activation in macrophages that allows for unrestricted replication of HCMV (177).

The host immune response to murine and human CMV has been studied extensively (reviewed in reference 19). Both specific as well as nonspecific immune surveillance mechanisms are employed in response to CMV. In murine CMV, natural non-specific immunity controls virus replication in acutely infected mice. At the inception of a CMV infection, natural killer (NK) cells provide the innate immunity by controlling virus until the specific immune arm arrives (125). Natural killer (NK) cells non-specifically provide a significant defense against CMV. Neutralizing antibodies do not play a significant role in the reduction of virus. However, when neutralizing antibodies are administered to animals, protection seems to occur naturally when newborns suckle immune mothers (125,118). In the presence of antibody, HCMV is transmitted, but the extent of disease is very mild. Cell-mediated immunity, via cytotoxic T-cells (CTLs) in particular, plays a large part in the clearance of virus in murine CMV. In humans, the presence of CMV-reactive CTLs, primarily specific to the pp65 and pp150 tegument phosphoproteins correlates with CMV protection (125,150). CD4+ T-cells also play a pivotal role in controlling viral replication in the salivary glands (80, 81, 125).

Antiviral therapies for treatment and prophylaxis of HCMV

HCMV disease ranges from mild to severe which is largely dependent upon the immune state of the host at the time of primary infection or reactivation following latency. Individuals armed with an intact immune system may develop cold symptoms or at most a mononucleosis-type syndrome. For a host immunosuppressed by AIDS or transplant therapy, a CMV infection could become life threatening. Several chemotherapeutic treatments have been utilized to treat the infection (reviewed in reference 15). These agents include leuokocyte interferon, interferon stimulators (i.e. pyran copolymer), transfer factor, as well as nucleoside drugs such as vidarabine, iododeoxyuridine, fluorodeoxyuridine, cytosine arabinoside and adenine arabinoside have been administered. However, due to an insignificant advantage clinically or extreme toxicity to the patient, these methods are no longer in use.

Acyclovir or ACV, active against other herpesviruses such as EBV, HSV-1, HSV-2, and VZV, was one of the first candidate treatments. The guanine nucleoside analog utilizes the viral thymidine kinase (TK) to phosphorylate acyclovir to its monophosphate form and cellular enzymes then act to phosphorylate the monophosphate form to its triphosphate form. The triphosphate form of acyclovir lacks a 3' hydroxyl group thereby blocking viral DNA replication by chain termination. Therapies used for CMV require a different mode of action because a viral thymidine kinase enzyme is not encoded by CMV (15, 166).

Two primary therapeutic agents in the treatment of CMV infection were ganciclovir (GCV) (DHPG- [9-(1,3 dihydroxy-2-propoxymethylguanine)]) and foscarnet (phosphonoformate). GCV is widely used to treat HCMV infections and has been used

in cases of HCMV pneumonitis, hepatitis, retinitis, colitis in AIDS patients, in addition to CMV infection in allograft recipients (166). The activity of GCV depends upon the virus-controlled initial phosphorylation in infected cells, however does not depend on the thymidine kinase of other herpesviruses (166). The UL97 open reading frame (ORF) in HCMV encodes a protein kinase that directs the phosphorylation of GCV to its monophosphate form (198).

Although GCV is widely used to treat HCMV infection, it has been associated with significant leukopenia and thrombocytopenia in some patients. As intravenous administration became toxic over time, GCV was used only in life-threatening infections of HCMV and an oral formulation later became available which decreased toxicity.

Foscarnet inhibits viral DNA polymerase function and recent evidence shows that it also inhibits hepatitis B virus polymerase and the HIV reverse transcriptase (15, 166). Only for life-threatening infections is foscarnet now used (15). Prolonged treatment of GCV or foscarnet may result in resistance. Resistant strains contain mutations in the DNA polymerase gene (UL54) or in the UL97 open reading frames which encodes a phosphotransferase required for phosphorylation of GCV to its active form (11, 109, 174, 198, 225). *In vitro* and *in vivo* evidence exists for increased effective treatment of GCV and foscarnet over any of the previously mentioned agents.

New targets for antiviral drugs are currently being developed. A serine protease (UL80 ORF) is required for the formation of nucleocapsids. Since all herpesviruses contain the ORF for a serine protease, a broad spectrum antiherpesvirus drug is a possibility. The DNA processivity (UL44) factor, functional in viral DNA replication, is also under consideration (reviewed in reference 15, 44).

Vaccines

Currently, no all-inclusive vaccine exists for protection against human cytomegalovirus. A live attenuated laboratory strain, the Towne strain, was used in the initial studies to prevent CMV disease in renal transplant patients (144). The virus was shown to be immunogenic in these patients, caused no CMV-associated disease, and failed to reactivate after the patients were immunosuppressed (114), (reviewed in reference 2). Either of the strains, Towne or AD169, was administered in normal seronegative individuals inducing both neutralizing antibodies and cell mediated immunity (132, 144). Protective immunity against a wild-type challenge, however, was unsuccessful although some effects were decreased. There has been some reservation in the use of a replicating viral vaccine for the prevention of HCMV disease. A primary concern is the possibility of vaccination of women of childbearing age with a replicating and/or teratogenic virus (15).

Recently, the focus of HCMV vaccination development has turned toward subunit vaccines. One aspect of development involves the production of HCMV proteins from recombinant expression systems. A large effort has been directed toward the CMV major envelope glycoprotein B (gB, UL55) (16, 125). The immunogenicity of gB has been demonstrated in experimental animals as well as in a study involving some humans (145, 179). Species specific protective immunity has been documented in mice treated with mouse CMV gB and guinea pigs treated with guinea pig CMV gB (55). The use of a subunit vaccine brings about some concerns because of the potentially different pathogenetic mechanisms in different populations (Reviewed in reference 15). For women of childbearing age, immunity which induces both a cellular response and the

production of neutralizing antibodies elicited by gB would mean protection from a possible congenital infection. In the case of allograft patients, CTL activity is closely associated with outcome, therefore a subunit vaccine may include a dominant CTL target such as pp65 (UL83). The pp65 tegument protein ORF (UL83) within expression vector is a candidate DNA vaccine. Mice infected intramuscularly with pp65 constructs resulted in a primarily humoral response in 60 % of mice injected (138).

The Virus Growth Cycle

General Aspects of the Replicative Cycle

The replication cycle for CMV is slow as compared to other herpesviruses. While HCMV requires 48-72 hours to produce detectable levels of progeny virus, HSV and VZV require 18-20 hours and 8-16 hours, respectively (15). The replication of HCMV can be detected as early as 15 hours postinfection in human fibroblasts. Unlike other herpesviruses, CMV fails to shut off host cellular metabolic processes. Instead, several studies have shown that CMV stimulates the synthesis of cellular protein, RNA, and DNA (29). It has been proposed early on that CMV DNA synthesis is dependent on cellular DNA synthesis (125,196). This issue has not yet been resolved.

Attachment and Penetration

HCMV glycoprotein makeup is much more complex than other herpesviruses. As the largest herpesvirus to date, the putative glycoproteins predicted far exceed any other herpesvirus. The amino-acid sequence of the laboratory adapted strain, AD169, shows greater than 54 ORFs with glycoprotein characteristics (24, 32). Clinical isolates such as

the Toledo strain contain an additional 13 ORFs which may encode glycoproteins. To date, however, only gB (UL55), gH (UL75), gL (UL115), gO (UL74), gp48 (UL4), GCF33 (UL33), gM (UL100) have been characterized as glycoprotein spikes (32, 72, 73). Each appears to have roles in HCMV entry into cells. Currently, only the envelope glycoprotein gB (gpUL55) has been extensively characterized (32, 125).

Several virus-cell interactions between HCMV and the infected cell have been defined with the majority of information derived using cultured fibroblast cell lines, although HCMV is capable of infecting distinct cell types of diverse lineages, including endothelial, epithelial, neuronal, glial, monocyte/ macrophage, and smooth muscle cells (reviewed in reference 5). *In vitro* studies show that human fibroblast cells express the viral receptor at significantly increased quantities over either epithelial or endothelial cell types. Also, fibroblasts are more efficiently infected *in vitro* than those cell types previously mentioned (135).

To infect cells, HCMV must first cross the host cell plasma membrane. Virus entry occurs at the cell surface in a two step process in which the virus must attach to the host cell followed by the penetration of the virus into the cell. Receptors are widely distributed on several different cell types (102, 135). Putative receptors have been found in both permissive as well as nonpermissive cell types. One cell surface component that is utilized by HCMV in at least two cell types is the heparin sulfate proteoglycans (HSPG) (33). gB and gpUL100 (a component of the gCII complex) have heparin binding ability. Heparin binding is loose and can be dissociated easily. This step is referred to as “low affinity binding”. Therefore, a more stable association with the cell surface is required via interaction with a second “high affinity” protein receptor (176). CD 13, a

cellular aminopeptidase, is a candidate for a “high affinity” binding receptor. The viral glycoprotein to which CD13 interacts is not yet known.

Two additional cell surface proteins have been implicated in the HCMV entry pathway, more specifically, at penetration. HCMV virions recognize a 30-34 kDa protein called annexin II belonging to a family of proteins involved in a number of membrane fusion events (32,226). A cellular 92.5 kDa protein is the proposed receptor for gH in the HCMV fusion process (84). Viral envelope proteins that may mediate binding to the cell surface remain uncharacterized and no direct genetic evidence of specific function for any HCMV glycoprotein is available to date (reviewed in reference 16). gB as well as other viral envelope glycoproteins are candidates for binding to heparin (33). Once the viral glycoprotein binds the cellular heparin sulfate receptor, an aminopeptidase called CD13 increases the stable binding before penetration (176). β_2 microglobulin may also act as a bridge between viral glycoproteins and the cellular receptor (32). Fusion of the viral envelope with the plasma membrane marks the initiation of penetration. In HSV, gH appears to be essential for viral penetration (85). Studies utilizing HCMV gH-specific monoclonal antibodies have indirectly shown that the HCMV gH glycoprotein is most likely functional in virion/plasma membrane fusion (85). gH participates in the entry process in concert with other glycoproteins. The gCIII complex, a 240-kDa protein, is a heterotrimeric disulfide-dependent glycoprotein complex (gH/gL/gO), consisting of three glycoproteins found in the virus envelope (72, 73). gH (gpUL75) is thought to function in entry and cell-cell spread, however, its cellular receptor is unknown (16, 125, 72, 73). gL (gpUL115) possesses a chaperone-type function required for the proper processing and targeting of gH (16, 72). gO (gpUL74) is the most recently characterized

component of the gCIII complex. The function of the tripartite complex in the viral life cycle is not well defined.

Immediate-early gene expression

In HCMV gene expression, the resulting proteins are produced in a very ordered and sequential manner, and as such are divided into three major classes based on the time of expression after viral infection in cell culture (36, 215, 216). In the sequential expression of the HCMV genes, the immediate-early (IE) genes are the first to be transcribed. The IE genes require no prior viral protein synthesis for expression but instead rely on the pp71 (UL82) virion transactivator as well as host cellular factors for expression (199, 200). The IE proteins serve as regulators of early gene expression. The IE genes are derived from various regions on the HCMV genome. In addition to the major IE locus (UL122-123), other genes expressed at IE times include UL36-38, TRS1-IRS1, and US3 (28, 99, 191, 192, 185, 188, 215, 221).

The major immediate-early promoter (MIEP), upstream of the major IE locus, functions in the activation and expression of the MIE gene region. The strong enhancer region within the promoter is quite powerful and is regulated by the 5' sequences containing repeated elements, that include cellular transcription factor binding sites within the MIEP (reviewed in 125). The repeated elements are referred to by their sizes as 16, 18, 19, and 21 bp repeats (14, 201). The corresponding proteins found to bind the repeats include NF κ B and CREB for the 18 and 19 bp repeats, respectively (125). The transcription factor YY1 binds the 21 bp repeat (97). Among the known or putative binding sites in the MIEP are the TATA box, AP1, CCAAT or C/EBP, SP1, TFIID/TBP,

p53, and retinoic acid. The immediate-early gene locus enhancer region of simian and murine CMV are almost indistinguishable from the HCMV MIEP (125).

The most abundant of the IE gene products arise from a single locus referred to as the major immediate early (MIE) region between 169 kbp and 175 kbp in the HCMV genome (reviewed in reference 125). Of the IE gene transcripts, two most notable (*ie1* and *ie2*) arise by differential splicing of a single transcription unit. Together, they share exons 1, 2, and 3 and the subsequent protein products [IE1 or IE72 (UL123) and IE2 or IE86 (UL122)] share the 85 N-terminal amino acids (186). The IE1 gene product is a 72 kD protein of 491 amino acids from exons 1, 2, 3, and 4 of the *ie1/ie2* gene locus (125, 186). The second gene product, from the IE2 gene region, is an 86 kD protein of 579 amino acids. It differs by virtue of a spliced transcript that includes exon 5 instead of exon 4 present in the *ie1* transcript (192, 193). From the *ie2* region, other transcripts are expressed such as the 55 kD protein of 425 amino acids generated as a result of intron removal from exon 5 (193, 187). A nonspliced transcript from within exon 5 produces a gene product of 338 amino acids only observed at late times in infection (148, 188, 193). Shortly after infection, the 72 kD and the 55 kD proteins are synthesized. A 38 kD, the 86 kD, and the 97 kD are produced at approximately 2.5 hours after virus adsorption. Additional genes with regulatory functions are expressed at immediate-early times. These include: TRS1 and IRS1 which are highly homologous and appear to have an overlapping function in HCMV DNA replication in transient assays (185). UL36, 37 products act as transactivators and are functional in DNA replication in transient assays (28). Both IRS1/ TRS1 and UL36-38 are members of the US22 gene family. US3 has

sequence similarity to glycoproteins and may have a role in immune escape during HCMV infection (28,221).

Functionally, the IE72 and the IE86 proteins play a major role in the regulation of the MIEP as well as in early promoter activation. Several studies support the roles of IE72 and IE86 in the positive and negative regulation of viral gene expression (38, 94). The role of IE72 appears to be in activating expression of the *ie1/ie2* enhancer, specifically by the self stimulation of the MIEP via the transcription factor binding site of NFkB (126). IE86 represses expression of the IE72/IE86 from the MIE gene locus by binding the cis-repression signal (CRS) (25). Although viral gene expression from the MIE locus is reduced at early times after infection, expression returns late in infection (188). Equivalent nuclear phosphoproteins with transactivation function have been observed in other herpesviruses (4, 86).

The expression of subsequent kinetic classes is also regulated through the cooperation of IE72 and IE86 proteins in conjunction with other transactivators, such as UL112-113 as well as the previously mentioned UL36-38 and IRS1/TRS1 (180, 181). While IE72 has been shown to transactivate via protein-protein interaction, IE86 binds other proteins as well as interacts with DNA directly to mediate gene expression. IE72 was found to associate with E2F-1, SP-1, and CRF-1 (61, 108, 113). IE86 plays a central regulatory role in the switch from IE to early gene expression (125). First, *ie1/ie2* expression is shut off by repression of the MIEP via IE 86 binding to the CRS as mentioned previously. IE86 is quite indiscriminate in its association with host transcription factors and its protein-protein interactions have been shown to result in the activation of early gene expression. Evidence for direct interaction of IE86 with TBP,

TFIIB, CREB, *c-jun*, and CBP has been established (18, 82, 103, 163, 165). Although no direct interaction has been found with IE72, IE86 has been shown to synergistically act with IE72 in the activation of early promoters (86). The N-terminus and the C-terminus have been found important in the transactivation by IE86 (125). The effects of IE1 and IE3 in murine CMV are analogous to IE72 (IE1) and IE86 (IE2) in HCMV (86, 119, 120).

Early Gene Expression

By current description, early gene expression requires the prior synthesis of IE gene products and occurs before viral DNA replication (180, 125). Early genes encode both structural as well as nonstructural proteins including enzymes involved in viral DNA replication (36, 215, 216). Numerous transcripts are produced during the early phase. However, only some of the gene products are evident in the early phase and others are often not visible until late in infection. This substantiates the thought that early genes are transcriptionally and posttranscriptionally regulated. A DNA negative mutant of HCMV, ts66, was used to demonstrate that early RNA transcripts have differential patterns of expression post viral DNA replication (186). From these studies, the early genes have been subdivided into three subclasses based on expression at early and late times post-infection. The first class of early genes is transcribed early and down-regulated after viral DNA replication (i.e.- 2.7 kb RNA; located in the long repeat of the genome corresponding to the *EcoRI* O and W fragments in strain AD169) (93, 186). The second class is expressed at constant levels throughout the course of infection [i.e.- (pol/UL54), (2.2 kb RNA/UL112-113)] (186). Lastly, the third class is expressed minimally early, but subsequent expression increases at late times [i.e.- (pp65/UL83,

encodes lower matrix protein), (1.2 kb RNA; mapped within the EcoRI-O fragment in the long segment of the viral genome in strain AD169) (186). A more recent set of studies has provided strong support for these observations by showing that the expression of three early RNA transcripts is quite different (180).

Numerous studies on several HCMV early promoters have demonstrated the specific mechanisms necessary to mediate promoter activation for early gene expression: in particular, the sequence requirements and the transacting factors involved (29, 38, 90, 95, 163, 164, 182, 184). Several assays have been widely used to determine the role of each of the HCMV major IE proteins in early promoter activation. One strategy comprises individual cDNAs for each protein that act as effectors under the control of a strong promoter such as the MIEP (38, 163, 184, 189). The target plasmid contains a hybrid promoter-reporter vector with the chloramphenicol acetyltransferase gene or the luciferase gene being more commonly used (23, 189). Transfected into fully permissive human fibroblast cells, a measure of promoter activation is assessed following cotransfection.

To date, experiments performed to study the transcriptional regulation of early gene expression propose that the IE86 protein alone or in conjunction with IE72, UL36, UL37, TRS1, IRS1, UL112-113 or US3 gene products (27, 88, 180, and 23) is required for early promoter activation. By the method described above, both IE72 and IE86 were shown to be required for maximal promoter activation on several early promoters (22, 38, 184, 189). The IE proteins activate the pp65 promoter through an octamer sequence (ATTTCGGG) (38). The polymerase promoter, also activated by IE transactivators, utilizes an inverted repeat 1 (IR1) element for its activation as demonstrated by a mutated

IR1 disrupting binding and adversely affecting its activation (90). More recently, Chau et. al. showed that the US11 promoter is not only synergistically activated by IE 1 and IE2, but is further stimulated in the presence of the phosphoprotein pp71 (23). The promoter utilizes the CRE and ATF sites which together cooperate to regulate the US11 promoter in HCMV infected cells (23). In addition, IE86 has been determined to be the major transactivator for at least three early promoters, the 2.7 kb, 1.2 kb and the 2.2 kb RNA promoters as the addition of IE72 alone did not significantly or only weakly increased the activation of the promoters (94, 103, 117, 184). When both IE72 and IE86 were transfected simultaneously, a modest increase in activation by 1-3 fold over IE86 activation was observed (180). Colberg-Poley et al. established the ability of other IE gene products such as US3, IRS1/TRS1, and UL36-38 to alter viral and cellular gene expression (28). UL112-113, TRS1 and IRS1, which are required in ori-Lyt viral DNA replication, are also transcriptional activators that augment IE1 and IE2 mediated activation of early promoters (75, 88). Although a significant level of promoter activity was observed in the presence of the IE transactivators, full activation to mediate the regulatory cascade takes place through the addition of a variety of cellular transcription factors.

Spector et al. have studied three early promoters (2.7, 2.2, and 1.2 kb RNAs) that exemplify differential expression patterns as mentioned previously (93, 95, 180, 184, 208). Through a series of well planned experiments, the promoters have been carefully studied to determine how each is regulated in early gene expression (180). The 2.7 kb RNA is detected at increased levels early in infection but decrease following viral DNA replication. The essential cis-acting sequences important for the regulation of the 2.7 kb

RNA were determined via the generation of deletions and point mutations within the 5' regulatory sequences. 5' deletion mutants resulted in a stepwise reduction in activity (93). The cis-acting sequences from -113 to -106 (a palindromic octamer sequence) were similar to the consensus binding site for MLTF/USF transcription factors (94). Point mutations in the MLTF/USF site decreased the inducible activity by 50 % in transfection assays using IE86 or HCMV as compared to wildtype. The identical mutations in gel shift analysis showed decreased binding affinity to the MLTF/USF site which thereby provided further evidence for its role in the 2.7 kb RNA promoter activation (94).

Unlike the 2.7 kb RNA, the 2.2 kb steady state RNA levels peak by 8 hours postinfection (hpi) and remain constant throughout the course of infection. The presence of alternative splice sites yields four RNAs of 2.1, 2.2, 2.5, and 2.65 kb (227). By deletion mutations, Rodems et al. determined that promoter activation of this early gene requires sequences from -113 to -59 containing a weak IE86 binding site as well as the consensus sequence for the cellular factor CREB/ATF (TGACGTCA) (152, 163). Site specific mutations confirmed sequences between -72 and -61 (CREB site) were critical for activation by IE86 (152). The promoter sequence also formed a complex with *in vitro* translated ATF2/CREB and antibodies directed against ATF/CREB family members showed one binding factor: CREB (152). Activation of the 2.2 kb RNA promoter was therefore facilitated by CREB (152, 163).

Lastly, the 1.2 kb RNA is expressed at low levels early in infection and increase at late times, hence, a member of the third class of early RNAs. The promoter is activated indirectly by IE86 through an API site (174). 5' deletion analysis revealed that

a TATAA like element (CATAA) at -30 and an AP-1 binding site at -75 are important for promoter activation (208). The key role of AP-1 and CATAA site was confirmed by the generation of specific point mutations in transient assays and gel shift assays. GST-fusion experiments in conjunction with coimmunoprecipitation experiments show that c-jun, junB, and ATF-2 bind IE86 (208).

Three different early promoters have been evaluated by similar approaches and clearly show that a combination of various IE proteins as well as cellular factors are necessary to activate the promoter sequences which regulate early genes. Transient expression assays in conjunction with protein-protein interaction studies in the identification of regulatory viral and cellular factors important for the regulation of early gene expression have correlated well (93, 94). HCMV early promoters are not ordinarily activated in uninfected cells or cells lacking the IE proteins. And further, it is clear that cellular transcription factors are insufficient for early promoter activation. Early promoters are not activated in HCMV infected cells until after the IE phase of gene expression begins. Therefore, it is the complex interplay between IE proteins and various cellular factors that are responsible for the vast diversity among early gene expression (190).

There have been numerous studies which address how HCMV transacting proteins influence the regulation of viral and cellular promoters (126, 186, 190). More recently, these studies have taken a leap toward a more accurate assessment of these regulatory events in the context of the viral genome. Jones and coworkers demonstrated that a region of the unique short (U_s) component of the HCMV genome is dispensable for growth in cell culture (79). From these findings, a strategy was developed to insert the

HCMV promoter-chloramphenicol acetyltransferase (CAT) gene constructs into the viral genome. In this way, sequences responsible for regulating HCMV at early and late times could be identified. RV134 is a recombinant virus which expresses the β -glucuronidase gene under the control of the 2.7E promoter (79, 96). The insertion of the HCMV promoter-CAT constructs into the transcriptionally barren intergenic region between the US9 and US10 open reading frames using the recombinant virus provided a more precise way to study the promoter cis-acting elements that regulate HCMV gene expression during a natural infection (96). Kerry et al, were able to extend the characterization of the UL54 promoter by inserting the polymerase promoter, mutated in the IR1 element and the ATF-1 element, into the recombinant virus (89, 90). UL54 regulation throughout the course of infection could then be assessed. The importance of the IR1 element in promoter activation at early times was confirmed, but the same element had no effect late in infection (88). The ATF-1 site, however, when mutated, reduced UL54 promoter activity at both early and late times (89). These data showed that *in vitro* and *in vivo* data do not always correlate. In 1998, Rodems assessed the function the UL112-113 early promoter in the context of the viral genome (152). The ATF/CREB site was functional at early times only, while the IE86 site was required for modulating the levels of RNA at early and late times (152). Recently, the US11 early promoter was examined by Chau in its natural context and found to utilize the CRE and ATF sites in the regulation of the US11 promoter in HCMV infected cells (23). Both elements were functionally important for promoter activation by IE proteins when assessed by deletion analysis. The mutagenesis of both sites revealed that full promoter activity requires the wildtype CRE and wildtype ATF sequences. Examined in the natural genomic context, mutation of the

CRE or ATF site resulted in a significant reduction in RNA levels. The simultaneous mutation of the CRE/ATF sites rendered RNA levels undetectable. A different system has been used to assess the HCMV US11 gene function in a natural infection utilizing the tet repressor system developed by Kim et al. (92). These studies demonstrated that a functional tet repressor was able to down-regulate expression from a US11 promoter containing repressor binding elements. Located in its natural position in the HCMV genome, the US11 promoter was modified with varying numbers of copies of the repressor binding sequence. This allowed for the conditional expression of viral proteins of interest (190).

In the chapters to follow, the UL98 early promoter has been extensively characterized. The 5' regulatory region of the UL98 alkaline exonuclease gene was isolated and the specific cis-acting elements functional in UL98 gene regulation were determined. The results demonstrated that a CRE and gamma IRE binding sites within the UL98 promoter have an important role in transactivation by the viral regulatory proteins from the HCMV MIE region. The transcriptional transactivator CREB was found to bind the UL98 promoter CRE site in DNA/protein interaction studies. CRE and ATF sites have also been utilized by early promoters for UL54, UL112-113, and US11. Therefore, the employment of CREB/ ATF family members in the regulation of HCMV early gene regulation appears to be a recurrent theme. This seems to reflect a general mechanism in early gene activation and is not specific for a particular early subclass.

Viral DNA Replication

Several proteins that play a role in CMV replication have been accurately predicted due to the similarities among other herpesviruses. Seven ORFs provide the necessary and sufficient viral proteins for viral replication (62). Among them are the origin binding protein (UL9) followed by the DNA polymerase (UL54), the processivity factor of the DNA polymerase (UL44), ssDNA binding protein (UL57), and the helicase-primase complex comprising three polypeptide subunits (UL105, UL102, and UL70) (6, 125). The HCMV genome is approximately 229 kilobase pairs (kbp) of linear double-stranded DNA. At approximately 4 hours after infection, the HCMV genome circularizes. Concatemers are formed and genomic inversions occur within the DNA. This is followed by the process of cleavage/packaging and seems to select the DNA termini derived from the S component rather than the L component into maturing capsids in one of four possible isomers (125). Viral DNA synthesis comes to a climax at 18-24 hours and again at 60-80 hours postinfection. Orilyt (the lytic phase replicator of HCMV) replication requires the use of several other trans-acting factors. These include UL54, UL57, UL44, UL105, UL102, UL70, UL122-UL123 (IE1-IE2), UL36-UL38, TRS1/IRS1, UL112-113, and UL84 (140, 141).

Late Gene Expression

Upon the initiation of viral DNA synthesis, late gene expression takes place producing primarily structural proteins. Due to the dependence on viral DNA replication and the need for a variety of transactivators produced at IE and E times, late gene expression requires a more complex regulation. Only a limited number of late genes

have been studied to determine the regulation of late gene expression. Genes currently classified as true late genes include the pp28 (UL99) phosphoprotein (38, 89, 96), viral glycoproteins (48), the 40 kD protein from the IE2 gene region (77), and the UL94 gene (224).

The pp28 promoter has been extensively studied and IE proteins were insufficient for promoter function (37). Protein products from the US3 (24, 221) and UL36-38 (24, 99, 200) gene regions also poorly activated the pp28 promoter. Therefore, additional viral proteins are required. No known consensus cellular transcription factor binding sites were found upstream of the CAP site. However, repeated sequences were found in the 5' promoter region in both the direct and inverted orientation. Perhaps only these sequences are necessary to regulate late gene expression (125).

Unlike early gene regulation studies, to study late gene expression requires the use of whole virus in transient assays; the use of IE and E transactivators in transient assays has proven ineffective (37, 125). As a result, little is known about the specific transactivators responsible for the initiation of late gene expression in HCMV. Stasiak and Mocarski characterized the ICP36 late promoter and found that TRS1, part of the US22 gene family, in combination with IE1 and IE2 proteins could activate this late promoter (185).

Late gene expression has been studied extensively in HSV. The gC promoter is activated early and no proteins are expressed until after viral DNA replication (69). This seems to be characteristic of true late promoters. Both HSV and HCMV promoters are activated at early times when expressed independent of the viral genome (37). Late gene

regulation studied using the HSV glycoprotein C (gC) was due to sequences near the 5' terminus of the gene (69, 154). The HSV gC gene contains sequences located in the 3' portion of the promoter that require the context of the viral genome for regulating gC as a late gene.

Virion Assembly

The assembly of HCMV begins late in infection with the MCP/PAP capsid proteins (which carry the nuclear localization signal), translocating from the cytoplasmic space into the nucleus. Nucleocapsids accumulate in the nucleus and form a nuclear inclusion often recognized microscopically by the typical "owl's eye" cytopathic effect(15). The nucleocapsids mature within a nuclear fibrillar network consisting of viral structural proteins and DNA. The formation of pre-B capsids into B capsids follows as the preformed capsids prepare to receive the DNA. The DNA is cleaved and packaged enzymatically in unit length from the replicating form. Capsids, now properly packaged with DNA, acquire the tegument proteins and become enveloped (50). While HSV envelopment takes place at the innernuclear membrane, cytoplasmic envelopment followed by egress has been reported for CMV and has been observed in CMV infected cells (50). An envelopment/ deenvelopment mechanism has also been suggested but has not yet been confirmed (125). The different pathways that virus can take during egress is not yet completely understood for the herpesviruses.

Herpesvirus Alkaline Nuclease

HSV-1 and HSV-2 viral alkaline nucleases were first described in 1968 by Morrison and Keir (128) and were expressed and mapped within the same viruses over 10 years later (129, 147). The mRNA has been mapped to a 2.3 kb mRNA and is a member of a group of unspliced 3' coterminal mRNAs (154). DNA sequence analysis revealed that the ORF, designated UL12, encodes a 626 amino-acid phosphoprotein of 85 kDa, which exhibits both exonuclease and endonuclease activities (47).

The HSV alkaline nuclease was not considered essential for viral DNA replication but may be functional in the processing of complex replicative DNA intermediates prior to their packaging into capsids (116, 217, 218). Herpesviruses contain large concatamers of DNA that are cleaved into unit-length genomes for packaging into preformed capsids. The DNA containing capsids are then transported from the nucleus to the cytoplasm, enveloped, and released from the infected cell. The HSV-1 UL12 gene product has been implicated in virion maturation, but its role in DNA processing is still not clear (217, 218). For HSV-1, it is thought that the nuclease functions in processing replicative DNA intermediates prior to packaging into capsids (116, 218). A deletion mutant (named AN-1) of the HSV-1 alkaline nuclease fails to induce alkaline nuclease activity in infected cells (167). The ability to synthesize viral DNA remains; however, the mutant fails to produce infectious virions efficiently and therefore, steps in genome maturation and encapsidation are defective (167). It has been proposed that the defect caused by AN-1 results in the inability to resolve or "debranch" recombination intermediates (115, 116, 167).

The UL98 gene of HCMV shares similar nucleic acid sequence homology as well as some amino acid sequence homology with the alkaline nucleases of other known herpesviruses (24). The exact function of the alkaline nuclease during infection is not clear. Sheaffer et. al. confirmed by *in vitro* activity that the alkaline nuclease of HCMV is the homologue of the HSV-1 alkaline exonuclease (169). More recently, an assessment of the biological role of HCMV UL98 revealed that UL98 AN can functionally substitute for HSV-1 AN (167). In addition, a defect in the HSV-1 AN is fully compensated by the HCMV AN when provided in trans (47).

The UL98 ORF shares a 3' coterminus with the ORFs of HCMV UL93-UL99 (1, 223). The HCMV UL98 ORF encodes an mRNA of 3.0 kilobases (kb) that is synthesized at early times of infection, and mRNA levels increase significantly following viral DNA replication (1). The UL98 gene product is a translated protein which migrates from 58-65 kD and is expressed at approximately five hours postinfection (1, 169). The translated protein is both phosphorylated and glycosylated and localizes to the nucleus late in infection and has been identified as a component of extracellular viral particles (169).

As previously mentioned, the early genes are divided into three subclasses (Early -1 or E1, E2, or E3) based upon the expression of the transcripts following infection. The complex regulation of the early phase has prompted extensive studies of the upstream 5' regulatory sequences necessary for the control of this complex class of genes. These studies have revealed the cis-acting sequences that confer promoter responsiveness to IE proteins as well as trans-acting factors that likely play an important role in the regulation of early genes. The HCMV UL98 early gene falls in the early

subclass that is expressed at low levels early postinfection, but rapidly increases after the onset of viral DNA replication (1, 223). The kinetics of UL98 gene expression is similar to two other E3 genes: the pp65 phosphoprotein and the 1.2 kb RNA gene product (38, 186, 208). For this reason, the 5' regulatory region of the UL98 gene was isolated and studied. The polymerase promoter has been studied extensively in the same laboratory and, as an early promoter (E2 subclass), was used as a basis of comparison and contrast (90).

To date, the HCMV UL98 5' regulatory sequences have been described in two separate papers. Wing and Huang determined the initiation start site, the TATA box, and the CAP site via primer extension and northern blot analysis as well as the consensus binding sites for SRF (5'GATGTCC3') at -152, E2F (5'TTTCGCCG3') at -127 and CREB/ATF (5'TACGTCAG3') at -82 relative to the RNA initiation site (223). As mentioned earlier, the CREB/ATF family of transcription factors are involved in the regulation of several viral early promoters (106). Both direct and inverted repeats were also found within the UL98 promoter (1). A DNAsis sequence data base search showed that elements for TCF-1 (5'TCTGTT3') at -59, SIF (5'GAGCTT3') at -43, and gamma interferon (5'CTGTATTT3') at -37 were present as well. The overall objective of the following studies is to determine how the HCMV UL98 promoter utilized its uniquely arranged response elements to optimally regulate the expression of this early gene. From these studies, we hope to obtain a clearer picture of the different mechanisms employed in early gene activation and regulation as observed in yet another early regulatory region.

CHAPTER II

SPECIFIC OBJECTIVES

The overall aim of this study was to determine the 5' regulatory elements which function in the regulation of the UL98 early gene. The UL98 promoter contains six putative *cis*-acting sequences upstream of the TATA box. In order to assess the functional role of each transcription factor binding site, an overlapping PCR mutagenesis approach was utilized for the generation of deletion mutations or substitution mutations at each site. The promoter regulatory sequences cloned upstream of a reporter gene were tested in transient transfection assays in the presence of viral immediate early (IE) transactivators or HCMV infection. Changes in promoter activity compared to the wildtype promoter due to mutations present provided insight into the *cis-acting* sequences that facilitate the process. The cooperative effort of two or more elements in UL98 promoter activation was examined by the generation of truncated promoters with the elements in question mutated in each promoter. To determine viral or virally induced transactivators that may physically complex with the cellular transcription factor binding sites, binding assays were carried out to identify the proteins that play a role in activation and facilitate UL98 promoter gene regulation.

The specific objectives of this study were:

- 1) To identify the UL98 5' promoter region relative to the putative TATA box motif and the CAP site at +1. The UL98 alkaline exonuclease promoter region was isolated as a 421 base pair DNA fragment. The strategy was to place the UL98 promoter sequences upstream of the chloramphenicol acetyltransferase (CAT) gene in a

reporter construct. The activation of the 5' regulatory sequences was tested in transient transfection assays in response to the immediate early transactivators IE72 and IE86 or HCMV. The IE requirement for UL98 promoter activation was also assessed using a prototypical early promoter of the UL54 polymerase gene.

- 2) To generate mutations within the UL98 promoter via restriction endonuclease digestion or overlapping PCR mutagenesis. Deletions were generated by digestion with *HindIII* (AAGCTT) and a unique restriction enzyme. By this method, sequence removal 5' to 3' in a stepwise fashion could be accomplished. In the absence of a downstream unique restriction enzyme site, the insertion of an additional *HindIII* site was feasible. For targeting a mutation, putative *cis*-acting elements were also changed using overlapping PCR to generate an *EcoRI* site (GAATTC). The resulting variant promoters were then tested for promoter activation in response to HCMV.
- 3) To determine the necessary transcription factor binding sites within the 5' regulatory sequences which confer UL98 promoter responsiveness to viral or virally induced proteins. Wild-type and mutant promoters were tested in transient transfection assays by cotransfection in the presence of IE expressing plasmids or in superinfection assays with HCMV.
- 4) To determine the transacting factors that form a complex with the transcription factor binding site by analysis of protein-DNA interactions by gel mobility shift assays. To identify which proteins, viral and/or cellular, bind the elements within the UL98 promoter, the DNA fragments of interest were radioactively end labeled and incubated with nuclear extracts containing DNA binding proteins. Competition studies were carried out to assess sequence specificity by the preincubation of excess

unlabelled probes with mock or HCMV infected cell nuclear extracts. To examine specific protein binding, antibodies specific for proteins suspected as binding UL98 sequence were employed in supershift assays.

CHAPTER III

MATERIALS AND METHODS

Cells

Primary Human Foreskin Fibroblast (HFF) cells were used for propagation of virus (Towne), transfection and superinfection assays, and the generation of nuclear extracts for gel mobility shift assays. Cells were grown in Minimal Essential Media Eagle (MEM) (Gibco BRL, Rockville, MD) supplemented with 0.03 % L-glutamine (Sigma, St. Louis, MO), 0.006 % Penicillin (Sigma, St. Louis, MO), and 0.01 % Streptomycin (Sigma, St. Louis, MO). Cells were incubated at 37°C, 5% CO₂. HFF cells were passaged on a weekly basis.

Virus

Towne, a laboratory adapted strain of HCMV, was used exclusively for the studies throughout. For superinfection assays, 10 PFU per cell was used. Frozen stocks of Towne were used at a titer of 5×10^7 PFU per milliliter. For gel shift analysis, cells were infected at 10 PFU per cell with a stock titer of at least 1×10^7 PFU per milliliter.

IE gene region plasmids and cDNAs

Plasmids able to express proteins from the HCMV major immediate early (IE) gene region 1 (pSVCC3), IE gene region 2 (pACC), and IE gene regions 1 and 2 (pSVH) were generated by R.M. Stenberg, Ph.D. and have been previously described (189). pSV0d was the vector used to construct the IE gene region plasmids. IE region 1 and 2 cDNAs were also generated by R.M. Stenberg; however, were placed in the vector pSL (an expression vector that contains a HindIII-EcoRI fragment of pcDVI, the HindIII-EcoRI of the pLI vector, and the simian virus 40 early promoter 5' to the *KpnI* site)

described in references (136, 189). pIE72kD cDNA and the pIE86kD cDNA contain the coding sequences of the major IE1 (72kD) and IE (86 kD) proteins, respectively (136, 189).

***Xba*I-C (pCBX8.1) plasmid**

pCBX8.1 is an 8.1 kb *Bam*HI to *Xba*I fragment of *Xba*I-C containing the pp28 gene region. From this construct, a digestion from *Pst*I to *Xho*I gave a subfragment of 621 bp and a subsequent *Bsr*FI digestion gave a subfragment of 421 bp which contains the UL98 promoter fragment.

pUL98CAT

To clone the upstream UL98 promoter, pCBX8.1, a plasmid containing the UL97 and UL98 open reading frames (ORFs), was digested with *Pst*I and *Xho*I to isolate a 0.6 kb fragment. Subsequently, a 421 bp (0.4 kb) fragment from -309 to +112 relative to the UL98 CAP site was isolated by digestion with *Bsr*FI (*Cfr*101I- isoschizomer). The digested products were separated by acrylamide gel electrophoresis. The fragment was repaired by Klenow fragment and 12mer *Hind*III linkers were added to the ends, cut, and purified through a 10% acrylamide gel. The fragment of 421 bp was cloned into the unique *Hind*III site of a linearized pSVOCATd upstream of the CAT gene.

pdH309CAT

pdH309CAT is devoid of the 3' *Hind*III site in pUL98CAT was used to generate the deletion mutants generated from the 5' *Hind*III site to a unique 3' restriction enzyme site or an inserted *Hind*III site. pUL98CAT was subjected to partial digestion with *Hind*III. The linear fragments were isolated on a 5% polyacrylamide gel, stained in ethidium bromide, and the linear products were isolated from the gel. The DNA was

removed from a gel slice by electroelution, precipitated with ethanol, dried, and resuspended in sterile deionized water (sdH₂O). For confirmation of correct isolated linear fragment, one tenth of volume of isolated DNA was run on a 1.0% agarose gel. The linear DNA fragment was Klenowed and religated at the unique *Hind*III site. Positive constructs for 3' *Hind*III knockout lost the ability to generate a 62 bp fragment when screened by digestion with *Hind*III and *Ava*I. The product containing only the 5' *Hind*III site was selected by restriction enzyme digestion with *Hind*III which now results in a linear fragment of 4.3 kb or 4,309 bp.

Deletion Mutants

To remove transcription factor binding sites 5' to 3', a linear pdH309CAT was digested with *Hind*III at -309 and a restriction enzyme for a unique downstream site. We initially generated three deletion mutants. pdH309CAT was digested from -309 to -194, -136, or -106. *Hind*III linkers were added to the fragments remaining (-194 to +3888, -136 to +3888, and -51 to +3888), which were then purified through a 5% acrylamide gel and prepared for religation. For the remaining deletion mutants, the *Hind*III restriction enzyme site was inserted between transcription factor binding sites using an overlapping PCR mutagenesis strategy. To generate each new *Hind*III site, four synthetic oligonucleotides (oligos) were generated and used as primers (see appendix III for specific primer sequences) for the *Hind*III insertions in two separate reactions. Two internal primers (b,c) contained the *Hind*III site to be substituted, and the external primers (a,d) consisted of vector sequences necessary to amplify the altered promoter region. In the first reaction (100 ul volume), primers [(a and b) or (c and d)] (20 picomoles each) were paired (one internal and one external complementary) in the presence of 1ul of

DNA template (1 nanogram per 1 microliter), 10x PCR buffer with Mg^{++} (Gibco BRL), 10 mM dNTPs stock (2 ul to give 1 mM of each dNTP per reaction), and Taq polymerase (0.5 ul at 5 units per ul) to insert and amplify the restriction enzyme sites into the sense and antisense DNA strands separately. The products of reaction one were subjected to Klenow in a fill-in reaction and incubated at room temperature for 15 minutes. In a second reaction, two overlapping products were then incubated under the same conditions with the two external 5' (a) and 3' (d) primers to amplify a double stranded UL98 promoter product with a *HindIII* site inserted. All PCR reactions were carried out under the same conditions in a thermocycler (Cetus). PCR conditions were set at 94°C/ 1 minute (denaturation), 55°C/ 1 minute (primer annealing), and 72°C/ 1 minute (extension) for 32 cycles followed by a soak setting of 4°C to maintain refrigeration temperature until processed. The final PCR products deleted to -85, -64, -34, and -28 were digested with *HindIII*, purified by electrophoresis, and cloned into pSVOCATd. Each deletion construct was tested in transient transfections in the presence of IE proteins or HCMV for changes in promoter activity as compared to the wildtype promoter construct (pUL98CAT).

Substitution Mutations

Mutagenesis of pUL98CAT construct by overlapping PCR (as described in the previous section: deletion mutations) allowed for the replacement of targeted wildtype sequence with the restriction enzyme site *EcoRI* (GAATTC). The restriction enzyme site *EcoRI* was selected to disrupt the cis-acting element located within the UL98 promoter region. This not only introduced a mutation at the desired site, but also provided a rapid means of screening for the mutation by restriction enzyme digestion in the final product.

To generate mutations, the linear pUL98CAT was used as a template. See appendix III for specific primer sequences used for the generation of each substitution mutation. One-tenth volume of the mutated PCR product was digested with *HindIII* or *HindIII* and *EcoRI* for confirmation of the *EcoRI* insertion. The remaining purified fragment was religated at the *HindIII* site -309 in the UL98 promoter and tested in transient transfection assays.

Sequencing of plasmid DNAs

Sequencing of the UL98 promoter was performed by the ALF DNA Sequencer utilizing the autoread kit (Pharmacia Biotech) and fluourescein-dNTP (F-dNTP) labeled primer/ quick annealing method. The sequencer is designed for the automated electrophoresis and analysis of sequencing reactions by the direct detection of fluorescently labeled DNA molecules. This differs from the traditional Sanger dideoxy sequencing method by the use of a fluorescently labeled primer or fluorescienated dNTPs instead of radioactive deoxynucleotides.

Electrophoresis is carried out in a vertical gel cassette. During electrophoresis, the fluorescently labeled fragments in each lane migrate downwards through the gel. The fixed laser beam passes through the glass light coupler located between the notched glass plate and the thermoplate of the gel cassette. The beam excites the fluorescently labeled DNA band and the light emitted is detected by photodetectors located behind the gel. The photodetector signals are collected, digitized and sent to the computer for storage and processing.

For each plasmid to be sequenced, 5 micrograms (μg) of closed circular DNA was mixed with 25 picomoles (pmol) of the fluorescein-dNTP labeled primer in a final

volume of 30 μ l. 1.5 μ l of 1N NaOH was added and incubated at 65°C for 5 minutes (min.). Tubes were transferred to 37°C and 1N HCl was added along with 2 μ l of annealing buffer. Tubes were then incubated at 37°C for 10 min. After a quick spin, the tubes were placed at room temperature for 5 min. F-dNTP labeling mix and T7 DNA pol was added and incubated at 37°C for 5 min. Following the addition of extension buffer and DMSO, 5.4 μ l of the mixture was added into 3 μ l of A,C,G, T mixes and incubated at 37°C for 5 min. The reactions were stopped by the addition of 6 μ l of stop solution and placed on ice. Before loading the samples onto the electrophoresis gel, the samples were heated for 5 minutes at approximately 90°C. Previously, the sequence of the UL98 promoter region and the initiation site was published for both AD169 (1) and Towne (185) strains of HCMV.

Transient transfection and superinfection assays

In transient expression experiments, pUL98CAT or its mutated derivatives were assessed by transient cotransfection with plasmids expressing one or both of the MIE proteins as previously described (38, 189) or by superinfection with the Towne strain of HCMV. Cells were seeded at 1×10^6 cells per 100mm dish (or 3.5×10^5 cells per 60 mm dish) on day one as determined by hemocytometer counts. On day two, using DEAE dextran method (191), cultures of HFF1 cells (at 80-90% confluency) on 100 mm dishes (or 60 mm dishes) were transfected with 5 (or 2) μ g of indicated reporter plasmid DNA. Cells were cotransfected with 5 (or 2) μ g of MIE gene plasmids (IE1 and IE2), followed by the addition of 1 ml of sterile Tris buffered saline (30 mM Tris-HCl pH 7.5, 150 mM NaCl) (TBS) and 0.5 mg of DEAE-Dextran (Pharmacia, Uppsala, Sweden). The DNA solution was added to cells washed prior with TBS and then incubated at 37°C for 30

minutes (rocking every 10 minutes). 10 ml of 100mM chloroquine (Sigma, St. Louis, MO) was added and the cells were incubated at 37°C for 3 hours. At 3 hours, media was removed and cells were then given fresh media and incubated at 37°C. Cells were then harvested 48 hours after transfection. For maximal transfection efficiency, a total of 10 µg of plasmid DNA was added per 100 mM dish initially seeded with 10^6 cells (38, 111). When activated by the Towne strain of HCMV, transfected cells were infected at 18-24 hours post transfection with 10-20 PFU per cell of HCMV and incubated for 2 hours. The inoculum was removed from cells and fresh media overlaid. Cells were harvested at 24, 48, or 72 hours post infection and extracts were processed.

Chloramphenicol acetyl transferase (CAT) assay

CAT activity was determined as previously described (38). 1 ml of TBS was added to transfected cells which were then scraped into microfuge tubes, spun down via centrifugation, and resuspended in 100 µl of 0.25M Tris pH 7.8. Cells then underwent three rounds of freeze-thaw followed by centrifugation to remove cell debris. The supernatants were transferred to new microfuge tubes, and heat inactivated at 68°C for 10 minutes. 20 µl of heat inactivated CAT extract were used per CAT assay reaction. A reaction mix also contained 0.1 uCi of ^{14}C -chloramphenicol (Amersham, Chicago, IL), 70ul of 0.25M Tris pH 7.8, 34 ul of sterile deionized water, and 20 ul of acetyl CoA (4mM). The reaction was incubated at 37°C for 30 minutes. 750 ul of ethyl acetate were added to stop the reaction followed by vortexing and centrifugation of each sample for 1 minute. The top aqueous phase was transferred to a new microfuge tube and dried down. The dried product was resuspended in 20 ul of ethyl acetate.

To determine the percent acetylation by chloramphenicol acetyltransferase (CAT), extracts were spotted onto thin layer chromatography (TLC) plates followed by autoradiography at -80°C for 16 -24 hours. The actual percent acetylation was obtained by applying TLC plates to phosphorimager (Molecular Dynamics) analysis.

In the experiments described above, CAT protein was not determined. However, μg quantities of protein could be determined in the Bradford Coomassie brilliant blue assay, which is accomplished by measurement of absorbance at 590 nm. This absorbance is compared to the absorbance of different amounts of a standard protein such as BSA (9).

Preparation of Nuclear Extracts

Human foreskin fibroblasts were infected with HCMV at 20 PFU/cell at confluence. After 1 hour adsorption, media containing virus was aspirated and cells were overlaid with fresh growth media. Extracts were prepared by a modification of the Dignam procedure (39). Cells were first washed with Tris-buffered saline (TBS) and harvested at a density of 5×10^6 cells per ml by scraping into buffer A containing 0.5% NP-40 and protease inhibitors [aprotinin $2\mu\text{g/ml}$, leupeptin $0.5\mu\text{g/ml}$, pepstatin $1\mu\text{g/ml}$, and phenylmethylsulfonyl fluoride (PMSF) 1mM]. Cells were lysed on ice by gentle pipetting for 10 minutes, and the nuclei removed by centrifugation at $1,500 \times g$. Nuclei were resuspended at approximately 1.5×10^7 nuclei per ml in buffer C containing protease inhibitors and incubated on ice with occasional gentle pipetting (every 10-minutes) for 30 min. Extracts were centrifuged at $50,000 \times g$, supernatant was removed, dialyzed against buffer D (1M HEPES pH 7.9, 1M KCl, 0.5M EDTA, 10% NP40, 100% glycerol, 1mM DTT, and PMSF) (This is a dialysis buffer containing KCl used to

normalize salt concentration during nuclear extraction, and the same buffer solution is also used for the preparation of nuclear extracts for both resuspension and dilution), and stored at -80°C.

CREB Protein

CREB protein (cat #: sc-4002) replaced the HCMV infected cell nuclear extracts and was incubated with the UL98 36 bp CRE specific radiolabeled probe in gel mobility shift assays. The CREB protein was purchased from Santa Cruz Biotechnology, Inc. CREB-1 bZIP (amino acids 254-327) was expressed in *E. coli* as a 10-14 kDa polypeptide mapping within the DNA binding and dimerization domain of CREB. The protein product was purified from bacterial lysates, and supplied as 50 µg purified protein in 0.1 ml PBS (1 µg/2 µl). The protein was assayed in a 1:100 protein dilution with Buffer D.

Gel mobility shift analysis

5-10 µg of nuclear extracts (10 µl) were incubated with 5 µg of poly (dI-dC) poly (dI-dC) (5 µl) and fold excess competitor DNAs for 15 minutes at room temperature in 50% buffer D. Radiolabeled probe was added (40,000 to 50,000 cpm, 0.5 to 1 ng of DNA), and the incubation continued for 30 minutes. Samples were subjected to polyacrylamide gel electrophoresis in one or one-half X Tris-borate-EDTA (TBE). Gels were dried and subjected to autoradiography (10).

Radiolabeling of DNA fragments or double-stranded synthetic oligonucleotides

DNA fragment

A 143 bp or 239 bp DNA fragment from the UL98 promoter region was isolated by restriction enzyme digestion using *NarI* and *MscI* or *NarI* and *AvaI* respectively, and

end-labeled with [γ - 32 P]-ATP and T4 polynucleotide kinase and purified with 1x STE buffer on a Nucletrap push column.

The 143 bp UL98 probe from -194 to -51 was isolated after restriction enzyme digestion of pUL98CAT with *Nar*I and *Msc*I and polyacrylamide gel electrophoresis.

The 148 bp nonspecific competitor fragment, lacking any UL98 specific sequences, was isolated in a like manner from the plasmid pSVOCATd.

Double-stranded oligonucleotides

Complementary single stranded oligonucleotides (10 μ g each) were incubated at 70°C for 30 minutes in an annealing reaction in 20mM Tris-HCl (pH7.5)-50mM NaCl-10mM MgCl₂ and the reaction mixture was subsequently allowed to cool to room temperature. The annealed product was then 5' radiolabeled with [γ - 32 P]-ATP and T4 polynucleotide kinase and purified by nondenaturing polyacrylamide gel electrophoresis. For each radiolabeled DNA reaction, 1 μ l was counted for cpm to determine amount to use per reaction in gel shift mobility analysis.

CRE- 36mer 5'CCATGCTGCACGAATACGTCAGAAAGAACGTGGAG3' and

3'GGTACGACGTGCTTATGCAGTCTTTCTTGCACCTC5'

TCF-1- 29mer 5'AAAGAACGTGGAGCGTCTGTTGGCCACTAT3' and

3'TTTCTTGCACCTCGCAGACAACCGGTGATA5'

CREm- 36mer 5'CCATGCTGCACGAAGAATTCAGAAAGAACGTGGAG3'

3'GGTACGACGTGCTTCTTAAGTCTTTCTTGCACCTC5'

TCFm- 29mer 5'AAAGAACGTGGAGCGGAATTCGGCCACGAG3' and

3'TTTCTTGCACCTCGCCTTAAGCCGGTGCTC5'

SIE/ γ IRE- 30mer 5'GGCCACGAGCGACGGGCTGTATTTATATAA3' and

3'CCGGTGCTCGCTGCCCCGACATAAATATATT5'

Sm- 30mer 5'GGCCACGAGCGAATTCCTGTATTTATATAA3' and

3'CCGGTGCTCGCTTAAGGACATAAATATATT5'

Im- 50mer

5'CCACGAGCGACGGGGAATTCTTATATAACGCCTTTCGGCGCACCACCAGC3'

and

3'GGTGCTCGCTGCCCCCTTAAGAATATATTGCGGAAAGCCGCGTGGTGGTCG5'

SmIm- 37mer 5'CCACGAGCGGAATTCGAATTCTTATATAACGCCTTTCG3' and

3'GGTGCTCGCCTTAAGCTTAAGAATATATTGCGGAAAGC5'

USI3m- 35mer 5'GCGTCTGTTGGGAATTCGCGAATTCGAATTCTTAT3' and

3'CGCAGACAACCCTTAAGCGCTTAAGCTTAAGAATA5'

To determine the HCMV infected cell protein(s) that bind specifically to elements within the UL98 promoter, the protein-DNA complexes formed in standard gel mobility shift analysis were assessed for further retardation by the addition of antibody(s) specific for proteins which are thought to bind the UL98 promoter elements. 5-10 µg of nuclear extracts (10 µl) were incubated with 5 µg of poly (dI)-poly (dC) (2 µl) for 15 minutes at room temperature in 50% buffer D. A ³²P radiolabeled probe of a specific promoter element was added and incubation continued for 30 minutes. Antibodies used include a CREB-1 mouse monoclonal (24H4B / corresponding to amino acids 254-327) which is non-crossreactive with other ATF/CREB transcription factors; ATF-1 mouse monoclonal (25C10G / epitope corresponding to amino acids 39-271) which

reacts with ATF-1 p35, CREB-1 p43 and CREM-1 of mouse, rat and human origin; or ATF-2 mouse monoclonal (F2BR-1 / corresponding to amino acids 350-505) which is noncrossreactive with other ATF/CREB transcription factors.

All antibodies listed above were commercially supplied by Santa Cruz Biotechnology, Inc. IRF-3 (rabbit polyclonal), generated in rabbits against IRF-3 amino acids 107-208 fused to glutathione S transferase (GST), was a gift from the laboratory of Michael David, Ph.D., UCSD, San Diego, Ca. For each antibody, 1 μ l (or 2 μ g) was added for an additional 30 minutes.

Cloning Methodology

Enzymes

Restriction endonucleases were obtained from New England Biolabs (NEB) (Beverly, MA.), Promega, (Madison, WI.), and Gibco BRL (Baltimore, MD). All enzymes were used according to instructions recommended by the manufacturer or as described in Maniatis (111). The Klenow polymerase fragment and the calf intestinal alkaline phosphatase (CIP) were ordered from Boehringer Mannheim Biochemicals, Indianapolis, In. T4 polynucleotide kinase was ordered from Pharmacia. T4 DNA ligase was ordered from New England Biolabs. Taq polymerase was ordered from Gibco BRL.

Restriction Endonuclease Digestion

Plasmid DNA construction was performed by methods previously described (111). Digestions were carried out in reaction mixes that contain 20 mM tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and the appropriate NaCl or KCl concentrations or in some instances, buffer supplied with the restriction endonucleases. One to five units of

restriction endonuclease per μg of DNA were used in each reaction. In the case of vectors requiring dephosphorylation of 5' ends, 20 units of high concentration calf intestine alkaline phosphatase (CIP) were added to the reactions. DNA fragments were then separated and purified by polyacrylamide gel electrophoresis. One fifth volume of loading dye (50% glycerol, 0.1 % bromophenol blue (BPB) 100 mM EDTA, pH 8.0) was added to the reaction prior to loading onto gel.

To separate and isolate products of restriction endonuclease digestion, reactions were loaded onto a 5% or 10% polyacrylamide gel run in 1x tris-borate-EDTA (TBE) buffer. Upon the completion of electrophoresis, the gel was stained with ethidium bromide (EtBr), visualized on a UV light box, and bands of interest were excised from the gel. To remove the DNA from the excised gel slice, electroelution was performed in 1/20x TBE buffer. Finally, DNA was ethanol precipitated in the presence of 200 mM NaCl and 100% ethanol at -80°C for 20 minutes. The precipitate was pelleted by centrifugation in a 4°C microfuge for 15 minutes. The pellet was washed in 70% ethanol and recentrifuged at 4°C for 5 minutes. Ethanol was discarded and the pellet was dried by vacuum and resuspended in an appropriate volume of sdH_2O .

Repair Ends of DNA Fragments

The pSVOCATd reporter-CAT construct contains a unique *HindIII* site just upstream of the CAT gene. To insert the wildtype or mutant UL98 promoter fragment, the ends of the DNA fragment were filled-in in a reaction that included dNTP, Tris pH 7.0, MgCl_2 , 1M DTT, 1-2 μl of Klenow polymerase. The reaction was incubated at room temperature for 30 minutes.

Ligation of Linkers onto DNA Fragments

Linkers were first phosphorylated as described in Maniatis (111), phosphorylated linker reaction contained 5mM ATP, T4 polynucleotide kinase (PNK), T4 PNK buffer, and incubated at 37 °C for 30 minutes. Linker ligations were performed using 50-100 molar excess of linkers to DNA fragments. The total DNA concentration was 10-20 µg/µl in 1x ligation buffer, and T4 DNA ligase. The reaction was carried out at 16°C for 16 -24 hours.

Ligation of DNA fragments with Vector DNA

Ligation reactions were performed essentially as described in Maniatis (111). DNA fragments were incubated with linear plasmid vector (pSVOCATd) DNA (1:1 to 4:1 ratio of insert to vector, 10-20 µg/µl DNA total) in 1x ligation buffer, 5 mM ATP, and T4DNA ligase for 16-24 hours. Following incubation, plasmid DNA was introduced into bacteria.

Amplification and Purification of plasmid DNAs

Competent cells used for transformation (HB101 or XL1 BLU) were prepared as described in Maniatis (111). Overnight small cultures of bacteria in Luria-Bertani (LB) media were subcultured into 250 ml LB media and grown to an absorbance of 0.4 to 1.5 at 550 nm. Cells were chilled quickly to 4°C, pelleted, and resuspended in 100 mM CaCl₂. Following a 30 minute incubation at 0°C, cells were pelleted in 100 mM CaCl₂ containing 15% glycerol, aliquoted into prechilled microfuge tubes and refrigerated on

ice at 4°C overnight. Bacteria were then quick frozen in 250 µl aliquots in a dry ice/ethanol bath and stored at -80°C.

For transformation reactions, 20 µl ligation reaction mix was incubated with 100 µl of competent cells for 30 minutes on ice. (4°C). The bacteria and ligation/competent cell mix was then incubated at 42°C for 2 and one-half minutes followed by 15 minutes incubation on the bench top at room temperature. The reaction mix was placed in 1 ml of LB media and placed in a shaking incubator for 1 to 1.5 hours. 100-200 µl of bacteria were plated onto an LB/agar plate containing 100 mg/ml ampicillin and incubated at 37°C overnight.

Screening of plasmid DNA

To screen for the desired plasmid, bacterial colonies were picked and placed in 2.0 ml cultures of LB media containing 50 ug/ml ampicillin and grown at 37°C for 16-24 hours. Plasmid DNA was isolated by a modified alkaline lysis method. 1.5 ml of each culture were pelleted, media removed and discarded, and bacteria suspended in 100 µl of solution I [25 mM Tris-Cl, 8% sucrose, 10mM EDTA, and lysozyme (5 mg/ml)]. Following a 5 minute incubation at room temperature (25°C), 200 µl of solution II (0.2N NaOH, 1% SDS) was added. Samples were mixed by inversion and incubated on ice for 5 minutes. 150 µl of solution III [3M Potassium Acetate (KAc), 11.5% glacial acetic acid] was added, mixed by inversion and placed on ice for an additional 5 minutes. Cellular debris and chromosomal DNA was pelleted. Plasmid DNA within supernatant was precipitated in 1 volume of 100% ethanol, dried and resuspended in sterile deionized water (sdH₂O). Plasmid DNA was sufficient for restriction enzyme analysis. RNase was added to each sample and incubated at room temperature to remove any RNA

present. To screen plasmid DNA, restriction endonuclease digestions were performed as previously described. Reaction mixtures were stopped with loading buffer [0.25% bromophenol blue (BPB), 0.25% xylene cyanol, and 30% glycerol]. Each was loaded onto a 1.0 to 1.5% agarose gel or a 5% acrylamide (short/thin) gel to resolve fragments smaller than 500 bps.

Large Scale Plasmid Preparation

To isolate large scale plasmid DNA, bacteria containing desired plasmid were grown until mid to late log phase (OD_{600} nm of 0.6 to 0.8) in 250 ml of LB media in the presence of ampicillin. Plasmid DNA was amplified with 1.7 mg of chloramphenicol per ml of culture. Bacteria was pelleted and plasmid DNA was purified by SDS-high salt extraction method. Bacteria pellets were resuspended in 7.5 ml 25% sucrose solution [10% (12.5 grams) sucrose, 50mM (10 ml) Tris-Cl] and 1.5 ml of 10 mg/ml lysozyme. After incubation on ice 5 minutes, 3 ml of 0.25 M EDTA was added. Following a second 5 minute incubation on ice, the mixture was warmed to room temperature and 1 ml of 12.5% SDS was added. The sample was mixed by inversion and incubated at room temperature for 15 minutes. After chilling samples to 0°C, 2.5 ml 5M NaCl was added and the mixture was incubated for 30 minutes. Samples were centrifuged at 19,000 rpm in a Sorvall ss34 rotor at 4°C for 45 minutes. The supernatants were transferred to a fresh tube, the volume elevated to 20 ml with dsH_2O , and treated with 100 μ l of 10 ng/ml RNAase A at 37°C for 1 hour. Following phenol-chloroform extraction, DNA was precipitated, pelleted, dried via vacuum, resuspended, and finally purified twice through cesium chloride gradients (190, 192, 193, 195, 201) to remove bacteria, genomic DNA,

and RNA. Each plasmid DNA was sufficiently pure for transfection into eukaryotic cells.

CHAPTER IV

RESULTS

Identification of the UL98 promoter relative to the putative TATA box and the CAP site at +1: cloning and characterization of the UL98 promoter in a CAT-reporter construct

The gene product of UL98 was previously identified as a 58 kDa protein homologous to the herpes simplex virus (HSV) alkaline exonuclease (1). Sequence analysis revealed direct and inverted repeats greater than or equal to 8 bp located upstream of the UL98 open reading frame (ORF). Consensus binding sites for ATF/CREB (-82), E2F (-127), and SRF (-152) were also found relative to the CAP site. A putative TATA box motif was present at -28. With the information gathered thus far, it was feasible to search for and isolate the promoter within this region. The plasmid pCBX8.1 contains an 8.1 kb *Bam*HI to *Xba*I fragment from *Xba*I-C containing the pp28 gene region. Initially, a 601 bp fragment containing the UL98 promoter region was removed by restriction enzyme digestion with *Pst*I and *Xho*I. This fragment included sequences from the UL97 ORF as well as the putative ATG of UL98. Therefore, it was necessary to digest the fragment further to include only the putative UL98 promoter. By digestion of the 0.6 kb fragment with the restriction enzyme *Bsr*FI, a 421 bp fragment from -309 to +112 (Figure 1) was isolated. The promoter fragment ends were filled-in with dNTPs by Klenow polymerase to blunt the ends and *Hind*III linkers were added. The UL98 promoter fragment was cloned into the pSVOCAT reporter construct at a unique *Hind*III site 5' to the chloramphenicol acetyltransferase (CAT) gene and designated pUL98CAT (Figure 2). Automated sequencing confirmed the proper

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-309  CCGGCGCGGC CTGCCGCGCG TTGGAGAACG GTAAGCTCAC GCACTGCTCC GACGCCTGTC TGCTCATTCT GGCGGCGCAA ATGAGCTACG
-219  GCGCCTGTCT CCTGGGCGAG CATGGGCGCG CGCTGGTGTC GCACACGCTG CGCTTTGTGG AGGCCAAGAT GTCCTCGTGT CGCGTACGCG
-129  CCTTTCGCGG CTCTTACCAC GAATGCTCGC AGACCATGCT GCACGAATAC GTCAGAAAGA ACGTGGAGCG TCTGTTGGCC ACGAGCGACG
-39   GGCTGTATT ATATAACGCC TTTCGGCGCA CCACCAGCAT AATCTGCGAG GAGGACCTTG ACGGTGACTG CCGCCAAGTG TTCCCCGAGT
+51   AACC GGGIACG CGGAACGTGA CGGTTGCTGA GGGGAAAGGC AACAGAGAAG GTACAAACCC A

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FIG. 1. Sequence of the putative UL98 promoter. The UL98 promoter was isolated by digestion with *Bsr*FI. A 421 base pair fragment from -309 to +112 was isolated and *Hind*III linkers were added to the ends. The elements specific for cellular transcription factors are underlined and represented by boxed abbreviations. The TATA box at -28 and the CAP site at +1 are indicated.

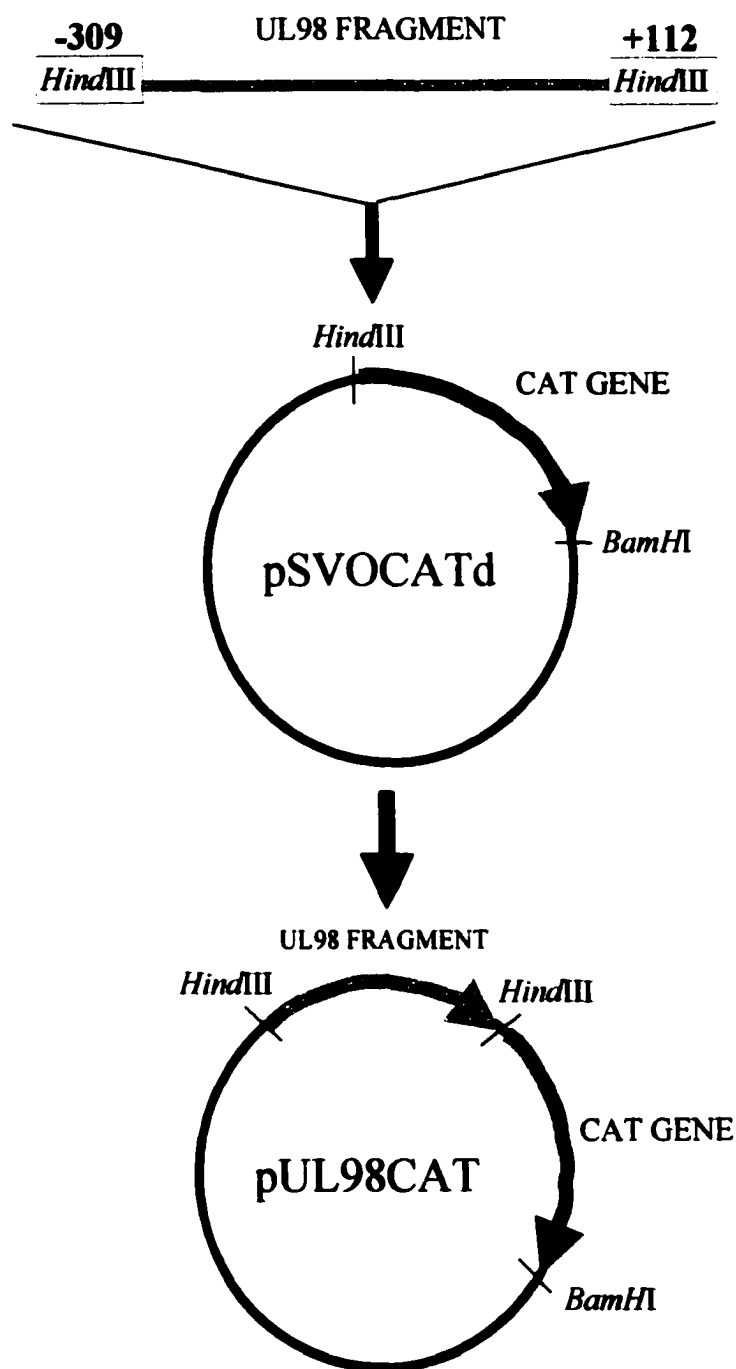


FIG. 2. Generation of the pUL98CAT construct. The promoter region of UL98 was obtained by digestion of the UL98 genomic construct with the restriction enzyme *Bsr*FI. The promoter fragment was isolated, *Hind*III linkers added to the ends, and cloned into the unique *Hind*III site of pSVOCATd upstream of the chloramphenicol acetyltransferase (CAT) gene to generate pUL98CAT.

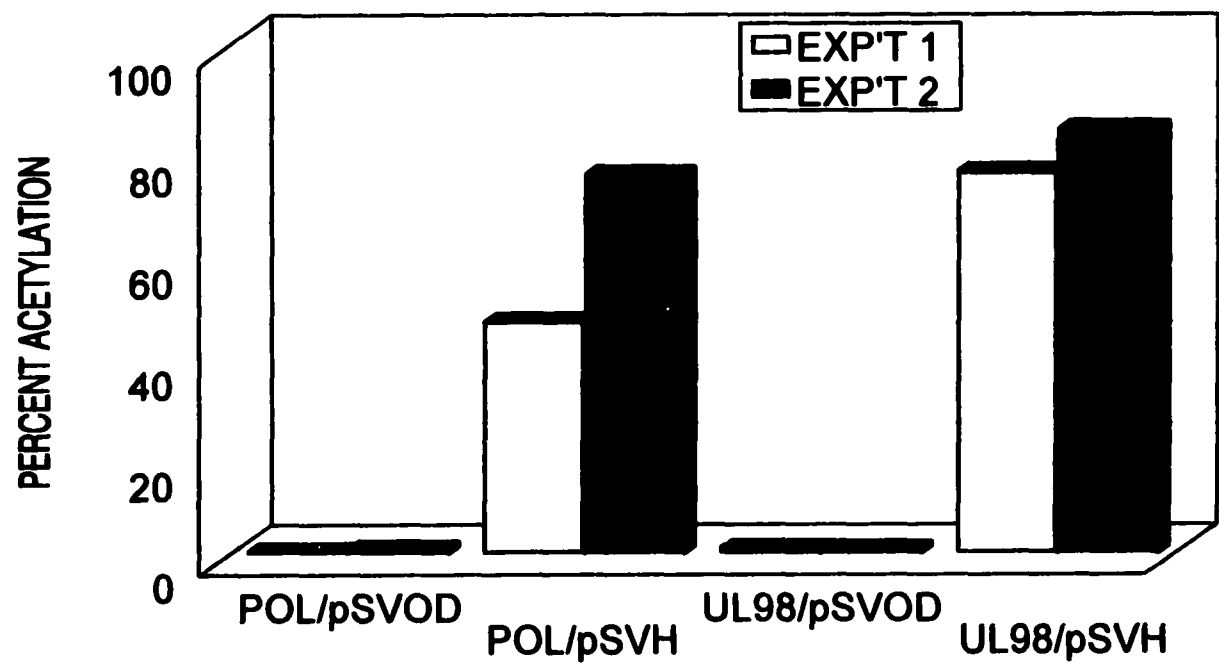


FIG. 3. Activation of early promoters ppolCAT and pUL98CAT by IE1 (IE72) and IE2 (IE86) gene products. Human foreskin fibroblast cells were transfected with 5 μ g of either reporter plasmid and 5 μ g of the plasmid expressing IE1 and IE2 (pSVH) or 5 μ g of the plasmid without IE1 and IE2 (pSVOD). Cells were harvested at 48 hours and assayed for the expression of CAT.

insertion and orientation.

The activation and subsequent regulation of early promoters require viral as well as cellular transcriptional factors. In transient transfection assays, HCMV promoters with early kinetics are activated by immediate early (IE) proteins (38, 181, 186, 189). To test the UL98 promoter for activation capability in the presence of HCMV IE proteins, pUL98CAT was cotransfected with a plasmid (pSVH) that expresses IE1/ IE72 (UL123) and IE2/ IE86 (UL122) into human foreskin fibroblast (HFF1) cells. An expression plasmid lacking the IE specific sequences (pSVOD) served as a negative control and was also cotransfected with pUL98CAT. The cells were harvested at 48 hours, processed, and assayed for CAT activity. The HCMV polymerase promoter is a well characterized early promoter strongly responsive to IE proteins in transient assays. pUL98CAT responsiveness to IE transactivators resembled the CAT activity observed from the polymerase promoter construct ppolCAT (Figure 3). The pSVOD plasmid, lacking IE1 and IE2 gene regions was unable to activate the promoter. Therefore, pUL98CAT responded well to IE proteins in transfection experiments and UL98 promoter sequences are activated.

Previous studies have documented that early gene activation requires the presence of immediate early (IE) transactivators (22, 38, 184, 189). Two such activators, IE72 and IE86 proteins, are necessary for the activation of various early promoters (pol, pp65, 2.2 kb RNA) in transient transfection assays. While several early promoters utilize both IE72 and IE86 for activation (UL54, UL83 promoters), others may only require IE86 for activation (UL112-113, UL4 promoters). The initial goal was to test the IE requirement for the activation of the UL98 promoter. pUL98CAT was cotransfected with pSVH (IE1

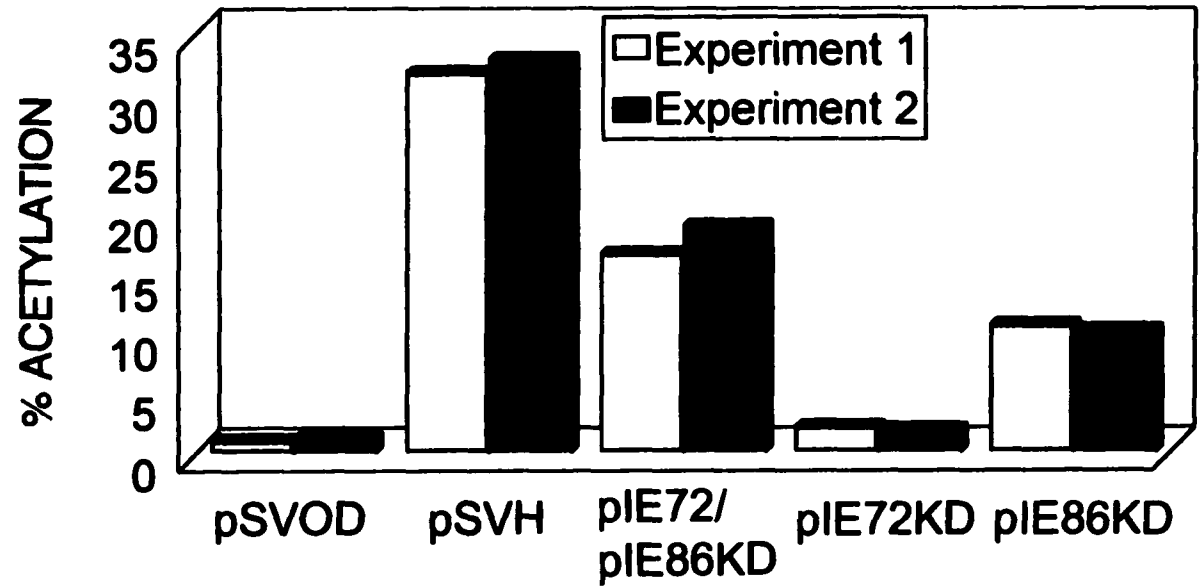


FIG. 4. Activation of pUL98CAT by IE1 and/or IE2. The ability of cDNAs IE1 and/or IE2 to stimulate the UL98 promoter was compared to the genomic construct (pSVH). pUL98CAT (5 μ g) was cotransfected with pIE72kD (5 μ g), pIE86kD (5 μ g), both pIE72kD and pIE86kD (2.5 μ g of each), or pSVH (5 μ g). Cells were harvested at 48 hours post transfection and assayed for CAT activity. Experiments were carried out in duplicate.

and IE2 administered in a single plasmid), pIE72KD (IE1 only), pIE86KD (IE2 only), or pIE72KD/ pIE86KD (IE1 and IE2 administered in separate plasmids). The cells were harvested and assayed for CAT activity as described above. Figure 4 shows that activation of the UL98 promoter requires IE1 and IE2 administered in a single plasmid (pSVH) for the most efficient activation. pIE72KD/86KD administered separately worked less well. The plasmid pIE86KD is able to activate the UL98 promoter (Figure 4), but to a lesser extent than with the cooperation of both IE proteins. pIE72KD alone scarcely activated the UL98 promoter. Therefore, IE72 and IE86 viral proteins together are required to efficiently activate the UL98 promoter. These data supports the IE transactivator model for the expression of early and late genes (186).

Deletion analysis of the UL98 promoter

The UL98 promoter contains consensus binding sites for ATF/CREB (cyclic AMP response element binding protein), E2F, and SRF (serum response factor) (223). Each of the proteins mentioned above plays an important role in the process of transcriptional activation. CREB is a cyclic AMP-regulated transcription factor defined as a sequence specific DNA activity (104). This protein binds to the cyclic-AMP response element (CRE) within promoters of cAMP inducible genes and mediates their induction in response to activation of the protein kinase A (PKA) pathway (63, 104). Several HCMV early genes utilize ATF/CREB sites for transcriptional activation. E2F is a cell cycle factor that binds to the product of the retinoblastoma gene, cyclin A, and adenovirus E4 (123). It can be induced by E1A and serum and functions in cell cycle regulation (123).

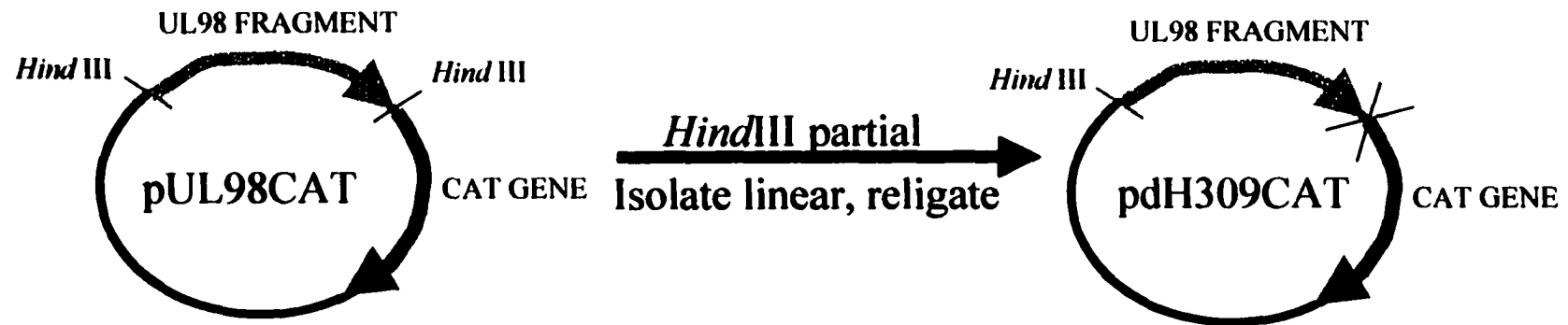


FIG. 5. Removal of the 3' *HindIII* site to generate pdH309CAT. pUL98CAT was partialled with *HindIII*. The linear products were isolated by acrylamide gel electrophoresis (5%). The purified product was religated followed by digestion with *HindIII* and *AvaI* to screen for the DNA product containing the disrupted 3' *HindIII* site. The altered promoter construct was then used for the generation of deletion mutations within pUL98CAT.

The serum response factor (SRF) is a 67 kDa protein (78). Binding to the SRE is essential for response to serum stimulation. SRE activity can be modulated by intracellular cAMP levels (168). In addition, a DNAsis sequence database search showed that elements for a putative TCF-1 (T-cell specific factor) (-59), an SIF (sis-inducible factor) (-43), and a gamma interferon (-37) are present as well.

The T-cell factor (TCF-1), part of the high mobility group (HMG) protein family, is a DNA binding protein functional in DNA bending (205, 206, 212). Found specifically in T-cells, closely related transcriptional regulators can be found in other cell types. Extensively characterized in the c-fos promoter, the sis-(PDGF) inducible factor (SIF), mediates induction by platelet derived growth factor (PDGF) (159, 160). In response to interferon α/β stimulation, SIF components (signal transducers and activators of transcription) bind the SIE (159, 160). Lastly, the interferon (IFN) gamma stimulates factors functional in the transcription of genes that function in the immune response. Several binding sites accommodate interferon gamma activated factors (34, 35). To determine the importance of each response element in UL98 promoter activation, it was necessary to generate deletions in a stepwise manner from the promoter's 5' end at -309 to the TATA box at -28.

Initially, the pUL98CAT construct required the inactivation of its 3' *Hind*III site at +112. In this way, deletions could be generated by digesting with the 5' *Hind*III site at -309 and 1) utilizing the unique downstream sites present or 2) inserting a 3' *Hind*III site at desired positions. The inactivation of the 3' *Hind*III site at +112 in the wild-type pUL98CAT construct was generated by partial digestion with *Hind*III. The linear

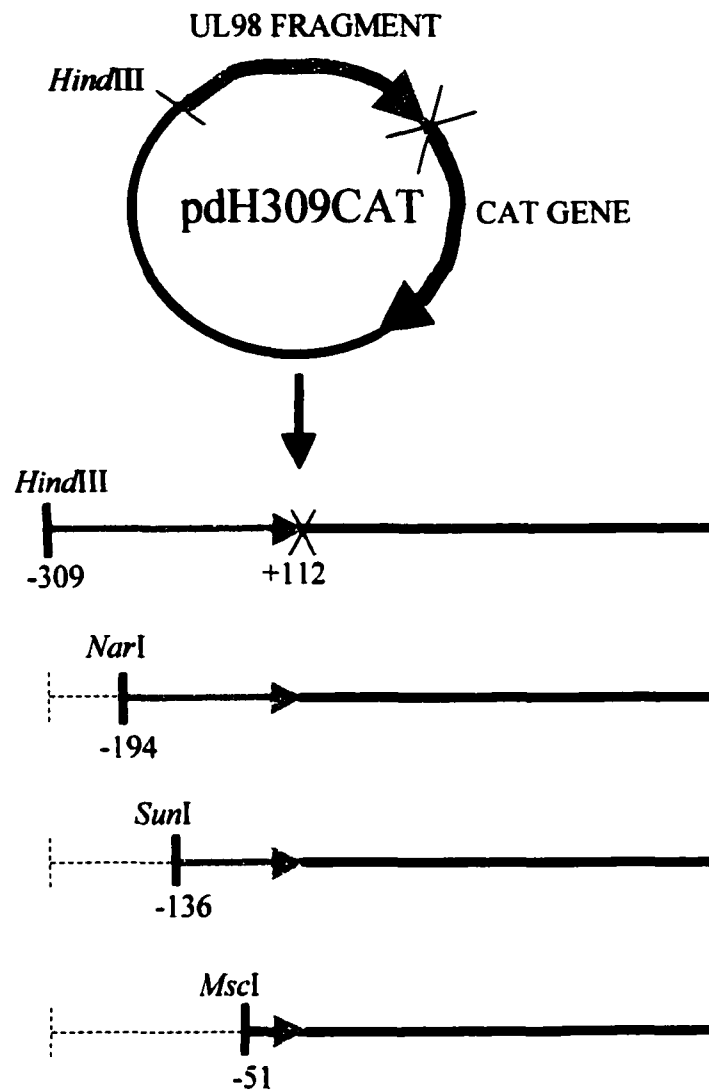


FIG. 6. Construction of UL98 deletion mutants utilizing pdH309CAT. The altered UL98 promoter construct was digested with *HindIII* and a second restriction enzyme site downstream to generated deletions from the 5' end of the promoter. The deleted site for each construct was discarded. The remaining sequence was isolated by acrylamide gel electrophoresis (5%), filled in with klenow, and religated with T4 DNA ligase. Variant promoter constructs include p194CAT [*HindIII* (-309) to *NarI* (-194)], p136CAT [*HindIII* (-309) to *SmaI* (-136)], and p51CAT [*HindIII* (-309) to *MscI* (-51)].

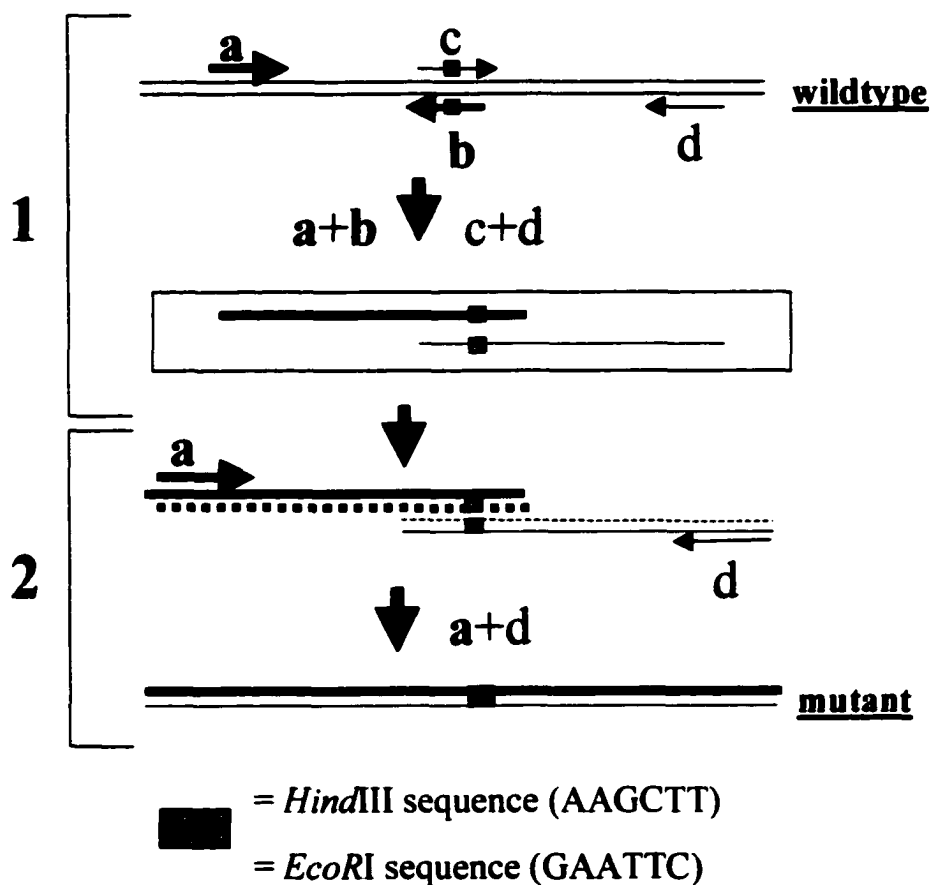


FIG. 7. A schematic representation of site-directed mutagenesis by overlapping PCR. The two solid lines represent the wildtype double stranded UL98 promoter. Four synthetic oligonucleotides (primers) are generated: two internal primers that contain the substitution mutation (grey box) and two external primers used to amplify each strand. In two separate reactions, a pair of primers (a+b or c+d) is incubated with the wildtype promoter (pUL98CAT) to generate two PCR products, which overlap at the mutation. To generate the mutated strands, oligos a and b were incubated with UL98 ppDNA as template to produce the 5' portion of the promoter with the mutation in the first reaction (1). Oligos c and d were treated in the same manner in a separate reaction to generate the 3' portion of the mutated promoter. The products of reaction one were subjected to klenow to remove additional A's attached by Taq polymerase. The products were then used as template in a second reaction (2) with only the external primers present to amplify the final full-length fusion/mutant product. All PCR reactions were carried out under the same conditions in a thermocycler (Cetus). PCR conditions were set at 94°C, 72°C, and 55°C for 32 cycles. For deletion mutations a *Hind*III site was generated, and for substitution mutations an *Eco*RI site was generated.

HindIII products were purified through a 5% acrylamide gel and religated. The products were then screened by restriction enzyme digestion with *AvaI* and *HindIII*. Positive constructs for the inactivated 3' *Hind III* sequence lost the ability to generate a 62 bp *AvaI* to *HindIII* fragment and were called pdH309CAT (Figure 5). To determine whether the altered 3' *HindIII* site affected the promoter activity, the pdH309CAT construct was tested in comparison to the parent pUL98CAT construct for responsiveness to IE proteins. In transient transfection assays, pUL98CAT or pdH309CAT was cotransfected with pSVH in HFF 1 cells, and at 48 hours assayed for CAT activity. The results showed that CAT activity in cells transfected with pdH309CAT resembled that of pUL98CAT, indicating that the inactivation of the 3' *Hind III* site did not negatively impact promoter activation by IE proteins (see Figure 10).

The pdH309CAT construct could now be used to generate a series of deletion mutations by progressively removing transcription factor binding sites 5' to 3'. Three deletion mutants were initially generated (Figure 6). pdH309CAT was digested with *HindIII* (-309) and a unique downstream restriction enzyme site to produce constructs that were deleted to -194 (*HindIII* to *NarI*), -136 (*HindIII* to *SunI*), and -51 (*HindIII* to *MscI*) in the UL98 promoter. *HindIII* linkers were added to the ends and the linear fragments were purified through a 5% acrylamide gel and isolated by electroelution. Each of the three fragments was religated at the deleted site to generate p-194CAT, p-136CAT, and p-51CAT. The constructs were subsequently tested in transient transfection assays.

Unique restriction enzyme sites within the UL98 promoter are randomly positioned, not conveniently separating each transcription factor-binding site. Therefore,

an overlapping PCR mutagenesis approach (Figure 7) was employed to strategically insert *Hind*III sites at desired positions between the response elements. Three elements between -136 and -51 [E2F at -127, the cyclic AMP response element (CRE) at -82, and the putative T-cell specific factor (TCF) element at -59], and two elements [the sis-inducible element (SIE) at -43 and the gamma interferon response element (γ IRE) at -37] between -51 and -28 required separation. Therefore, it was necessary to utilize a method for the insertion of restriction enzyme sites. The pdH309CAT construct contains a single 5' *Hind*III site at -309. By the insertion of a second *Hind*III sequence at a downstream site, subsequent digestion with *Hind*III would result in the removal of other transcription factor binding sites.

Briefly, to generate each new *Hind*III site, four synthetic oligonucleotides (oligos) were generated and used as primers for the *Hind*III insertion in two separate reactions (Figure 7). Two internal primers (b,c) contained the *Hind*III site to be substituted, and the two external primers (a,d) consisted of vector sequences necessary to amplify the altered promoter. In the first reaction, complementary primers [(a and b) or (c and d)] were paired (one external and one internal) to insert and amplify the restriction enzyme sites into the sense and antisense DNA strands separately. In a second reaction, two overlapping products were then incubated with the two external 5' (a) and 3' (d) primers to amplify a double stranded UL98 promoter product with a *Hind*III site inserted (Figure 7). While new internal primers were generated for each *Hind*III insertion, the same external primers (a and d) could be used. The final PCR product was digested with *Hind*III and thereby deleted to -85, -64, -34, or -28. For each deletion, the larger *Hind*III fragment in tandem with the CAT-reporter construct was purified by electrophoresis

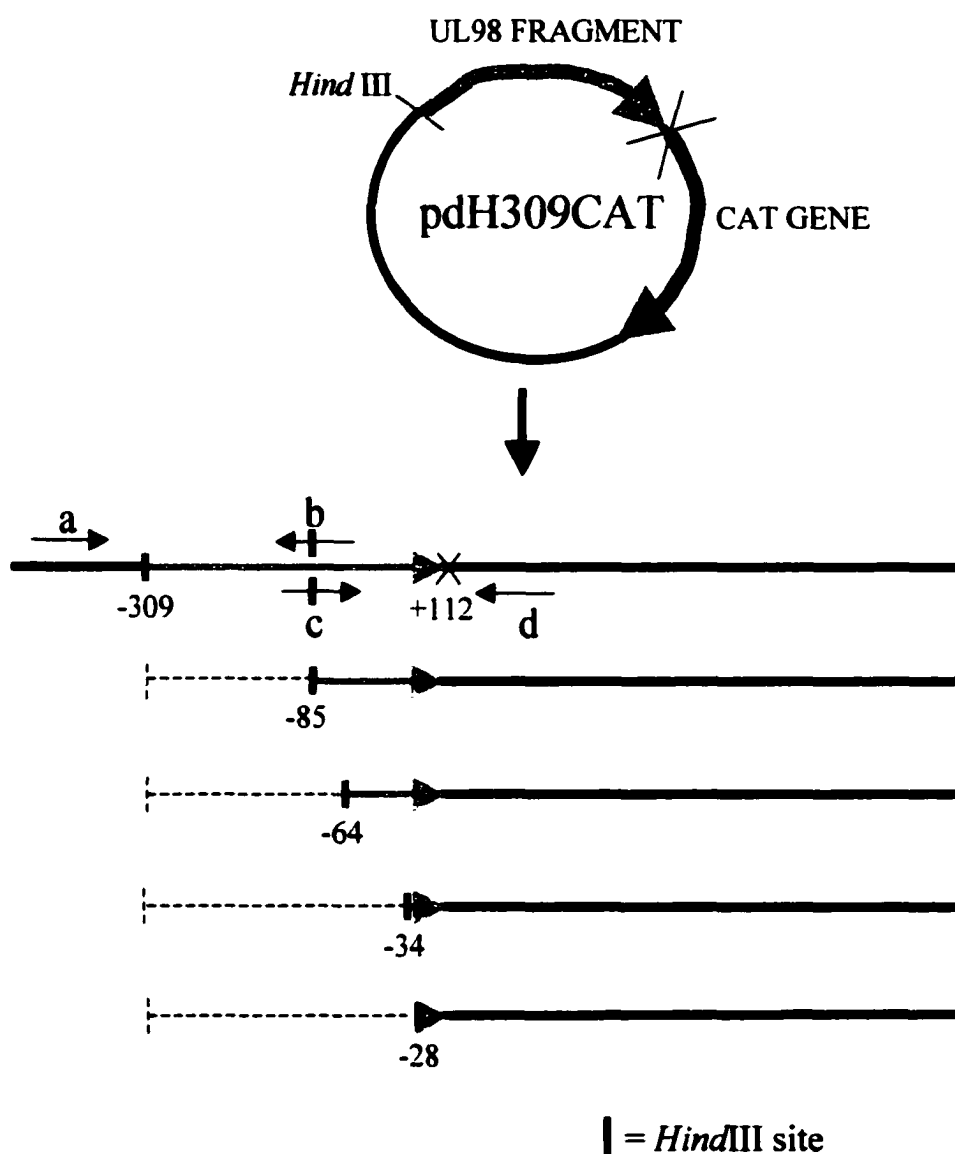


FIG. 8. Insertion of the *Hind*III site for the construction of deletions in the UL98 promoter. pdH309CAT acts as a template for the two reaction process of overlapping PCR mutagenesis. Reaction one utilizes four primers (two internal and two external) for the insertion of the *Hind*III sequence. Reaction two requires the use of two external primers for the amplification of the mutated product. Final PCR products are purified through a 5% acrylamide gel and inserted into the unique *Hind*III site of pSVOCAT. Deleted promoter constructs are as follows: p85CAT (-85), p64CAT (-64), p34CAT (-34), and p28CAT (-28).

through a 10% polyacrylamide gel and religated (Figure 8).

The importance of each element could be assessed because each promoter construct now contains one less transcription factor binding site with each deletion toward the TATA box. Each construct was then evaluated in the presence of IE regulatory proteins to determine the impact of each transcription factor binding site on UL98 promoter activation. In transient transfections, each deletion construct p-194CAT, p-136CAT, p-85CAT, p-64CAT, p-51CAT, p-34CAT, and p-28CAT (Figure 9) was cotransfected in the presence of IE proteins with pSVH, harvested 48 hours after and processed for determination of CAT activity.

Results in Figure 10 show that while promoter constructs deleted to -194, -136, -85, and -64 were activated to levels similar to wildtype, the promoter deleted to -51 was drastically reduced in promoter activity. Promoter constructs deleted to -34 and -28 (p-34CAT and p-28CAT) were also activated at or slightly below wildtype levels. p-51CAT was the only deletion construct able to abrogate UL98 promoter activity. From these data, sequences within a 13 bp (-64 to -51) region were found critical for the activation of the UL98 promoter (Figure 10).

The T-cell factor (TCF) element (5'-TCTGTTGG-3') is positioned from -59 to -54 on the negative strand (3'-CCAACAGA-5') and may therefore serve as a critical component in the regulation of the UL98 promoter. It was anticipated that the removal of all transcription factor-binding sites 5' of the TATA box would significantly reduce promoter activity as found with other early promoters. It was surprising to observe that, in transient assays, deletions downstream of -51 but upstream of the TATA box resulted in promoter activity similar to wildtype (See Figure 10). This observation suggests the

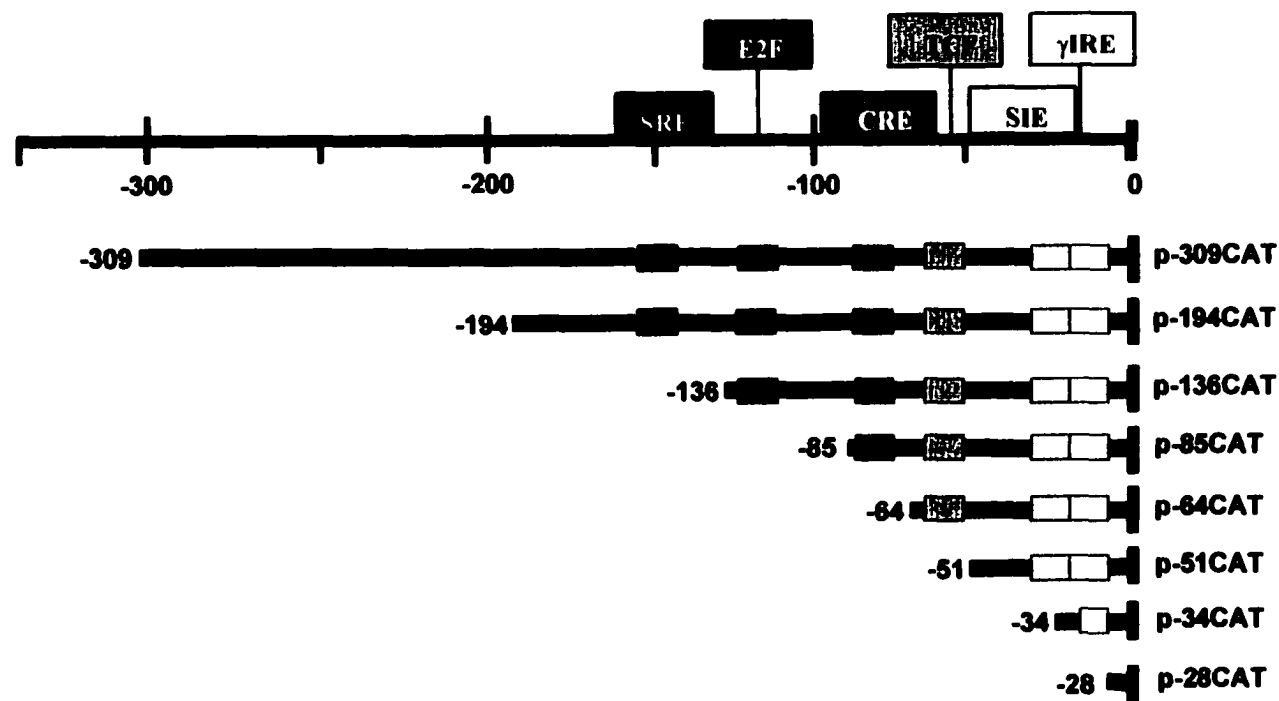


FIG. 9. UL98 promoter deletion mutants. The elements specific for cellular transcription factors are underlined and represented by boxed abbreviations. Each deletion removes a cis-element from -309 toward the TATA box at -28. Deletions were generated by restriction enzyme digestion or overlapping PCR mutagenesis and confirmed by automated sequencing. Variant promoter constructs are named to the right of each illustrated construct.

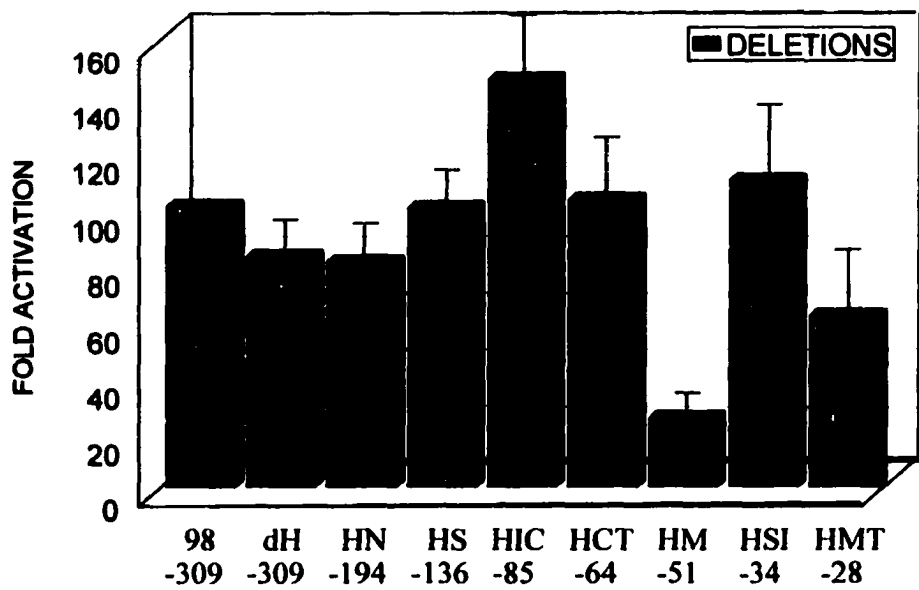


FIG. 10. Responsiveness of promoter deletion mutants to IE proteins. Individual promoter construct DNAs (5 μ g) were cotransfected with 5 μ g of pSVH into human fibroblasts, harvested at 48 hours, and assayed for CAT activity. Results were converted to fold activation compared to wildtype as 100 percent. This data shows that the region important for promoter activity lies between -64 and -51.

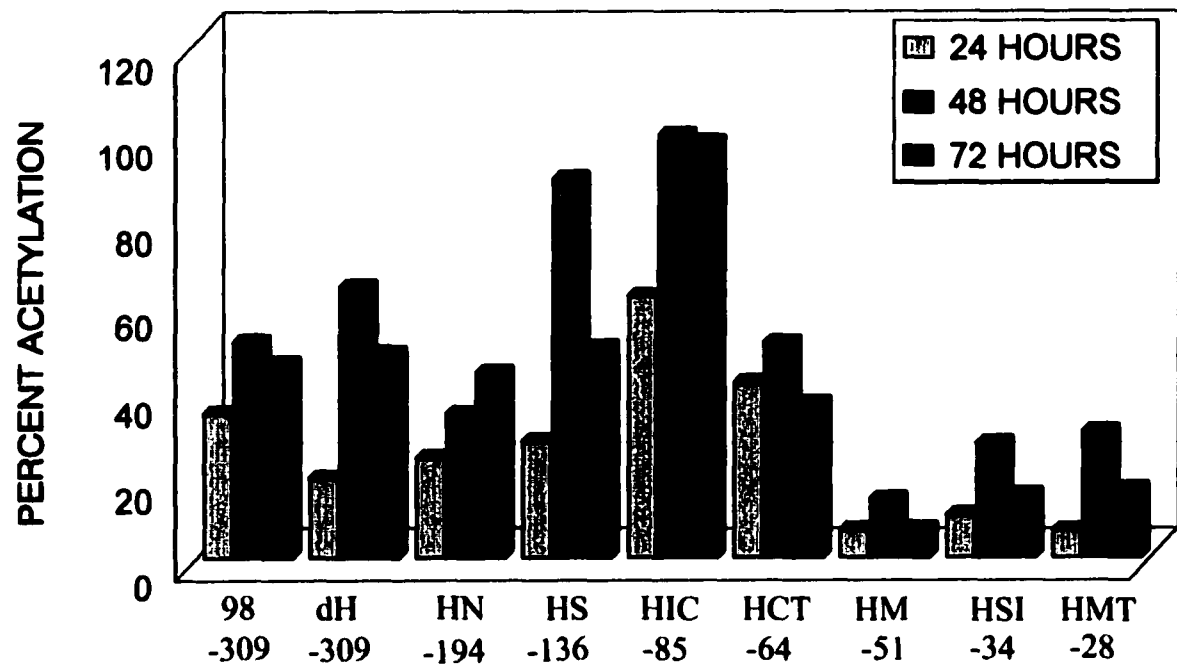


FIG. 11. UL98 promoter deletion mutants activated by HCMV infection. Activation of the UL98 parental and variant promoters were assessed by transfection of each plasmid (10 μ g) into human fibroblast cells followed by superinfection with the Towne strain of HCMV (10 pfu per cell) at 24, 48, and 72 hours after infection. Percent acetylation was quantitated by phosphorimager analysis.

presence of a possible repressor element located between -51 and -28.

In order to assess UL98 promoter activation in the presence of all viral or virally induced transactivators, HCMV was used to infect cells following transfection with each promoter construct. Thus far, data in the characterization of the UL98 promoter stems from transient assays only showing activation in the presence of IE viral proteins.

Promoters are regulated by viral as well as cellular factors. Therefore, it was necessary to utilize HCMV in place of constructs which produce only IE proteins. To assess activity of UL98 wildtype or mutant promoters in the presence of HCMV, the deletion mutant promoters were each transfected into HFF1 cells followed by viral superinfection with HCMV (10 pfu per cell). The cells were harvested at 24, 48, and 72 hours post infection and processed for CAT activity. At each timepoint, deletions from -194 to -64 were activated to the same relative level as the wild-type UL98 promoter (Figure 11).

However, the construct deleted to -51 resulted in a greater than 60% decrease in promoter activity at all time points. -34 and -28 deleted promoters were also decreased in activity compared to wildtype. The promoter deleted to -85 responded to HCMV infection well above the UL98 wild-type promoter at 24, 48, and 72 hours. These data provide further evidence that the sequences important for UL98 promoter activation lie upstream of -51.

In summary, the UL98 promoter 5' regulatory sequences were deleted in a stepwise fashion. A model has been developed to illustrate how each deletion mutant responded to the IE gene products IE72 and IE86 or HCMV (Figure 12). According to the deletion analysis data, promoters deleted to -64 (-194, -136, -85, -64) and tested in the presence of IE72 and IE86 resulted in promoter activation equivalent to or above the levels of the UL98 wild-type promoter. The construct deleted to -51, however, had a

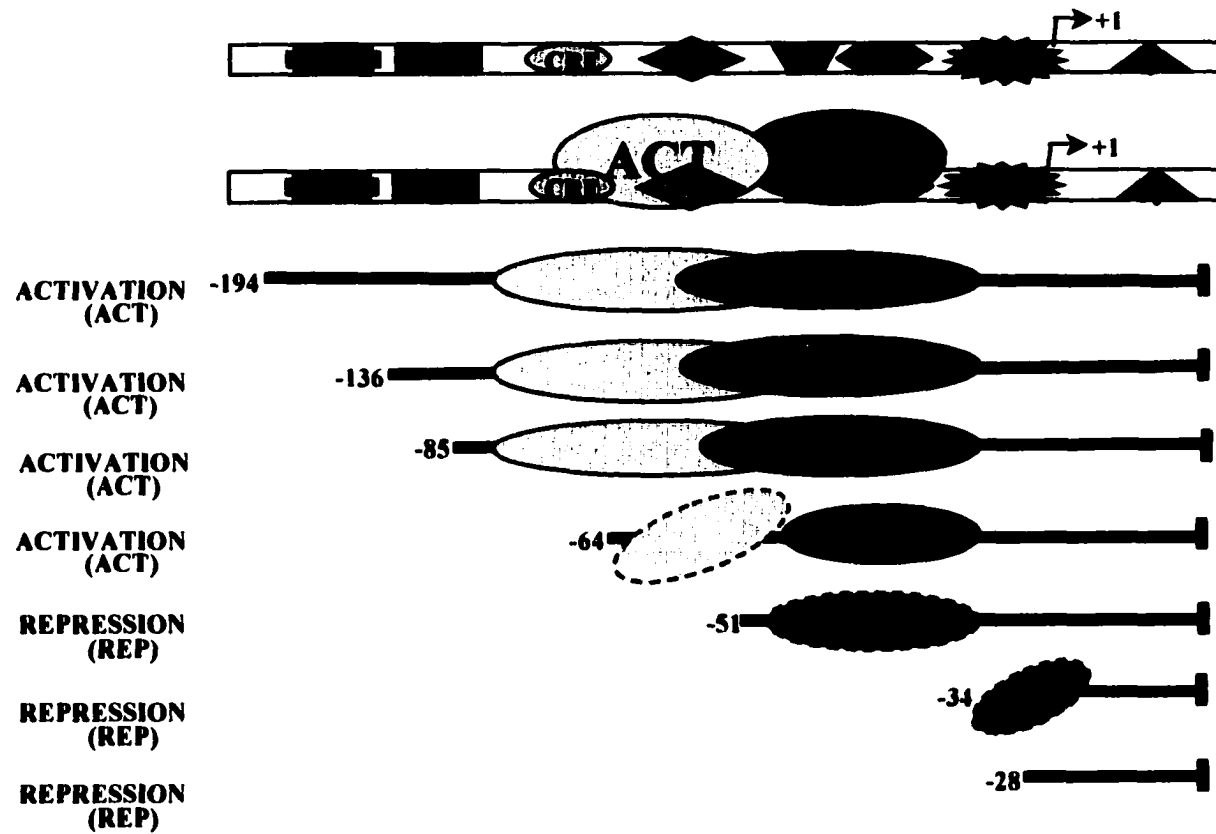


FIG. 12. Model of UL98 Promoter Activation. Based on deletion analysis data, the top line represents the UL98 promoter with designated cis-acting elements. Proposed transacting factor(s) functional in activation are illustrated on the second line as activators (ACT) which may bind the promoter for activation. Each deletion mutant is depicted as a solid black line containing putative transacting factors that may bind in response to the deletion generated. The phenotypic response observed in HCMV superinfection is labeled to the left of each deletion mutant.

significant decrease in promoter activity. It is, therefore, suspected that the sequences between -64 and -51 play a role in promoter activation. By the removal of additional sequences in promoter constructs deleted to -34 and -28, promoter activity was restored to wildtype levels as shown in transient data (See Figure 10). In this instance, downstream sequences appear to become dominant and a repressed phenotype is observed. When superinfecting with HCMV, in the presence of all transactivators, promoters deleted to -34 and -28 act similar to -51 also showing decreased promoter activity. From these data, it appears that the addition of virally regulated proteins following HCMV infection prevents the return of activation which was observed in the -34 and -28 promoter mutants in response to IE proteins.

Mutational analysis of specific response elements in the UL98 promoter

Analysis of the deletion mutants provides an introductory assessment of the region between -64 and -51 as important for UL98 promoter activation. The removal of response elements may lead to loss of promoter context and therefore may prevent the promoter from functioning in the manner observed when all elements are present and properly spatially arranged. The next series of studies were performed to determine which element(s) was necessary for UL98 promoter activation. It was, therefore, necessary to alter each response element individually while leaving the remaining promoter intact lengthwise.

To determine the precise transcription factor binding site(s) important in the regulation of the UL98 promoter, substitution mutations were generated in the response elements between -85 and -28 (CRE, TCF element, SIE, and γ IRE). Because members

UL98 Mutants in the Full-length Context

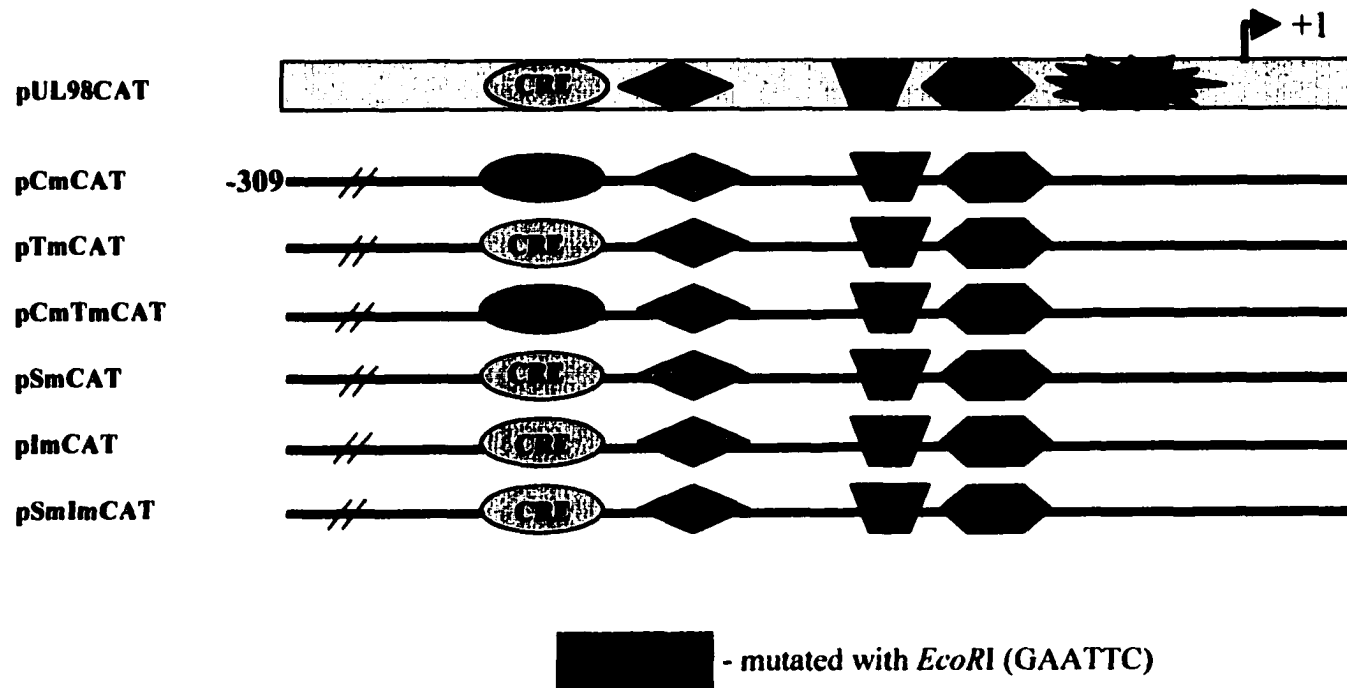


FIG. 13. A schematic representation of the UL98 promoter mutants. Specific sequences within the UL98 promoter were mutated by site-directed mutagenesis utilizing the overlapping PCR method. Substitution mutations of various promoter elements were changed to *EcoRI* restriction enzymes sites (noted as empty grey box). The wildtype promoter sequence labeled pUL98CAT shows each element within the promoter as well as the location of the TATA box and the CAP site (+1).

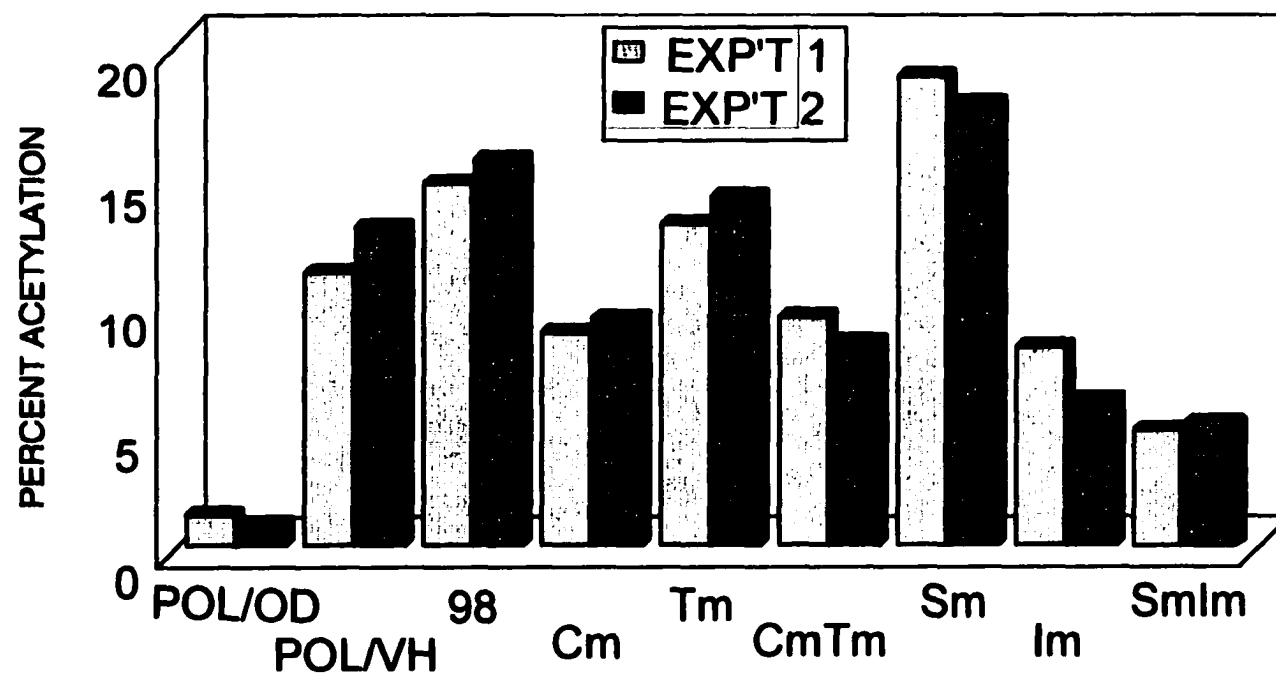


FIG. 14. Activation of site-directed promoter mutants in the presence of IE proteins. Mutant construct DNAs (5 μ g) were cotransfected with 5 μ g of pSVH into human fibroblasts, harvested at 48 hours, and assayed for CAT activity. Duplicate experiments are represented in this figure. All substitution mutations were sequenced to verify the presence of the substitution in the proper position and correct orientation.

of the ATF/CREB family had been implicated in the activation of other early promoters (75, 148), the CRE sequence may also play a role in UL98 promoter activation and was altered into a mutated CRE (Cm). TCF-1 element was also mutated (Tm), considering its location within the 13 bp region (-64 to -51) which was found necessary for UL98 promoter activity in deletion analysis. A mutation was also generated in the 5' portion of SIE (Sm) (-43) or γ IRE (Im) (-37) because the elements responsive to interferon (IFN) vary widely and have been implicated in promoter activation and repression. A total of four substitution mutations were generated: CREm, TCFm, SIEm, and γ IREm.

Mutagenesis of the pUL98CAT construct by overlapping PCR allowed for the replacement of targeted wildtype sequences with the restriction enzyme site *EcoRI* (GAATTC) (see Figure 10). This restriction enzyme site was chosen because, prior to mutagenesis, the promoter region contained no *EcoRI* sites. As previously described in site-directed mutagenesis, two internal oligonucleotides were generated to substitute each element (CRE, TCF element, SIE, and γ IRE) with an *EcoRI* site (GAATTC). In the first reaction, each internal oligonucleotide was paired with a complementary external primer to alter the targeted element. The product from the first reaction served as the template to amplify the mutated fragment in a second reaction using only the external primers.

Insertion of an *EcoRI* (GAATTC) site not only introduced a mutation at the desired site, but also provided a rapid means of screening for the mutation by restriction enzyme digestion in the final product.

The mutated PCR product (Figure 13) was digested with *HindIII* and the purified fragment cloned into a reporter-CAT plasmid. Each individual promoter was cotransfected with pSVH (Figure 14) or transfected followed by superinfection with

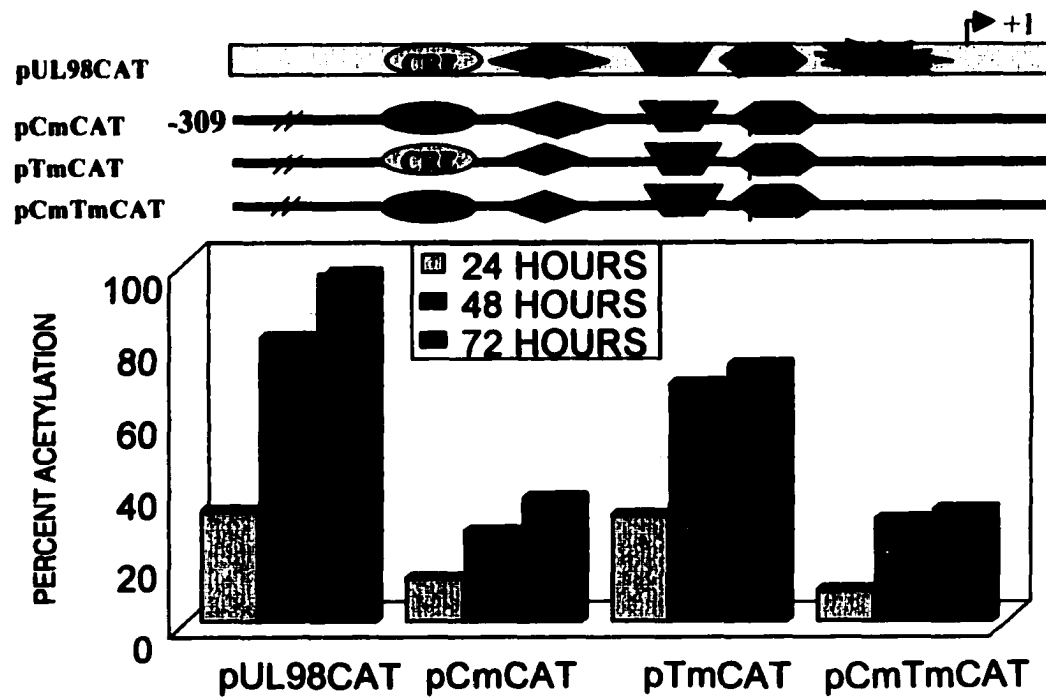


FIG. 15. Time course of activation of pUL98CAT and mutants (CRE/TCF element) following superinfection with HCMV. Wildtype and mutant promoter/CAT constructs were transfected into human fibroblasts on day one and superinfected with HCMV (Towne strain) at 10 pfu per cell on day two. Cell extracts were harvested at 24, 48, and 72 hours post infection and examined for CAT activity. pCmCAT and pCmTmCAT, both containing a mutated CRE site were reduced significantly at each time point.

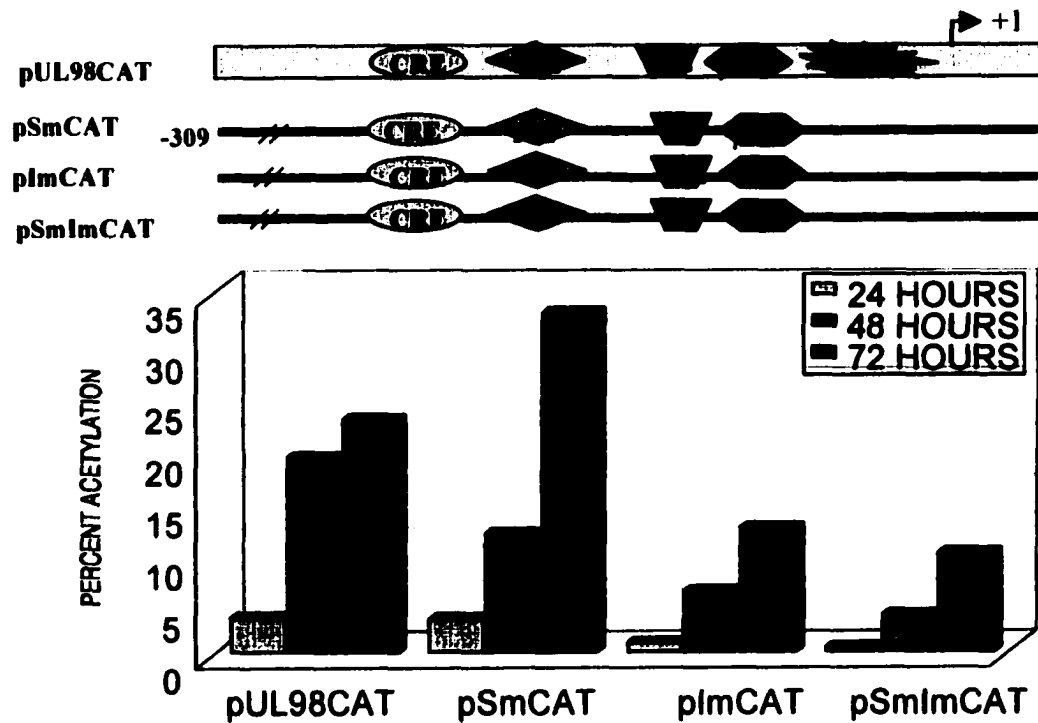


FIG. 16. Time course of activation of pUL98CAT and mutants (SIE/γIRE) following superinfection with HCMV. Wildtype and mutant promoter/CAT constructs were transfected into human fibroblasts on day one and superinfected with HCMV (Towne strain) at 10 pfu per cell on day two. Cell extracts were harvested at 24, 48, and 72 hours post infection and examined for CAT activity. pImCAT and pSmImCAT, both containing a mutated γIRE site were reduced significantly at each time point. pSmCAT was reduced at 48 hours only.

HCMV (Figure 15,16) and tested in transfection assays. Results from these studies showed that in transient assays, the promoters mutated at CRE and γ IRE (pCmCAT and pImCAT) were reduced in promoter activity only slightly as compared to wildtype, while mutations generated at the TCF-1 element and SIE (pTmCAT and pSmCAT) resulted in activity similar to wildtype (Figure 14). From these results, it appears that mutating a single element only affects promoter activation minimally, meaning two or more elements may cooperate to maximally activate the UL98 promoter. In experiments in which each individual promoter was transiently transfected followed by superinfection with HCMV, promoters containing a mutated CRE or mutated γ IRE were reduced greater than 50% (Figures 15, 16). These data show that the CRE and γ IRE play a role in UL98 promoter activation, however the cooperative regulation between elements remains a possibility.

To determine whether CRE and TCF-1 element cooperate, a double mutation was generated. Previous studies have shown that promoter activation via TCF-1 must rely on flanking sequences such as CRE (20, 49). Perhaps CRE and TCF-1 element binding proteins cooperate in UL98 promoter activation. A double mutation was also generated in the SIE and γ IRE within a single construct. Two weak IFN response elements side by side have functioned together to generate a strong IFN binding site (107, 142). Perhaps the SIE and γ IRE are present in tandem to function in a similar capacity. Each double mutant construct was examined in transfection experiments and assayed for CAT activity. Because the promoter containing the CRE/TCF double mutation responded to HCMV superinfection much like the CRE only mutant (Figure 15), CRE appears functional in activation supporting UL98 promoter regulation by CRE. The SIE/ γ IRE double

mutation was significantly reduced similar to the γ IRE mutation in the UL98 promoter (Figure 16). In superinfection assays, only the promoters containing a single or double mutation with CRE or the γ IRE significantly reduced promoter activation at all time points, supporting a role for CRE as well as γ IRE (Figures 15, 16) in UL98 promoter gene regulation. Therefore, neither CRE and the TCF-1 element nor the SIE and γ IRE appear to cooperate in UL98 promoter activation.

From the data above, the cyclic AMP response element (CRE) and the gamma interferon response element (γ IRE) are major participants in UL98 promoter activation. Transfection assays revealed that within the 13 bps between -64 and -51, lies the TCF-1 element from -59 to -52 and these promoter sequences are necessary for promoter activation. The TCF-1 protein, which binds the TCF-1 element, has been shown to act not as a transactivator, but instead as a facilitator of DNA bending allowing widely separate sites to be brought into close proximity for the activation of the promoter. While some transcriptional activators also bend DNA, others lack the ability to bend DNA once bound. Perhaps TCF-1 binding sequences aid in the activation of UL98 promoter, and influence the role of flanking sequences such as CRE and γ IRE in the UL98 promoter/enhancer region.

Two promoter constructs, p-85CAT and p-64CAT, were shown to have activation similar to wildtype (pUL98CAT) in the presence of IE regulatory proteins or HCMV. A deletion to -85 consistently resulted in activation at or above the wild-type promoter. A deletion further to -64 was slightly decreased in promoter activation but still remained close to wild-type levels. Each of these truncated promoter constructs were efficiently activated in the presence of viral regulatory proteins (see Figure 11). If the cyclic AMP

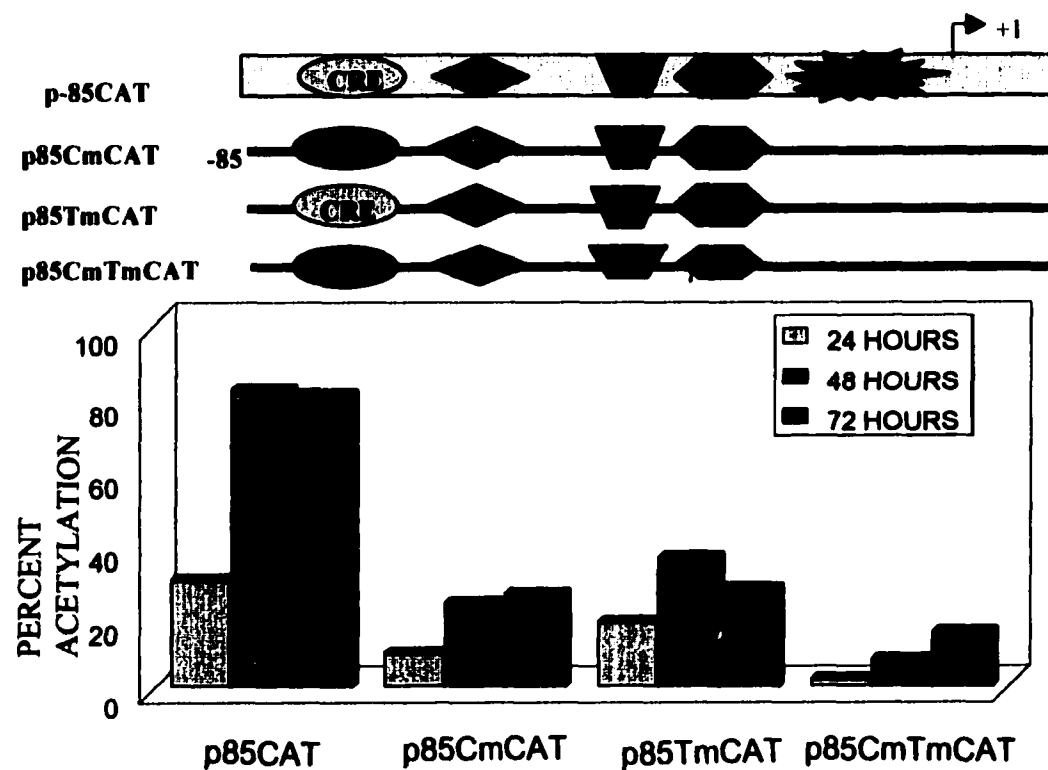


FIG. 17. CRE/TCF element mutations in the context of the -85 truncated promoter following superinfection with HCMV. To determine how the mutated elements function in the -85 minimal promoter which has activity at or above wildtype levels, constructs were mutated at CRE and TCF-1 element. 4 μ g of each mutated CAT-reporter constructs, labeled to the far left, was transfected into human fibroblasts on day one, followed by superinfection with HCMV at 10 pfu per cell on day two. Cell extracts were harvested at 24, 48, and 72 hours and processed to determine UL98 promoter activity. Graphed data is expressed as percent acetylation.

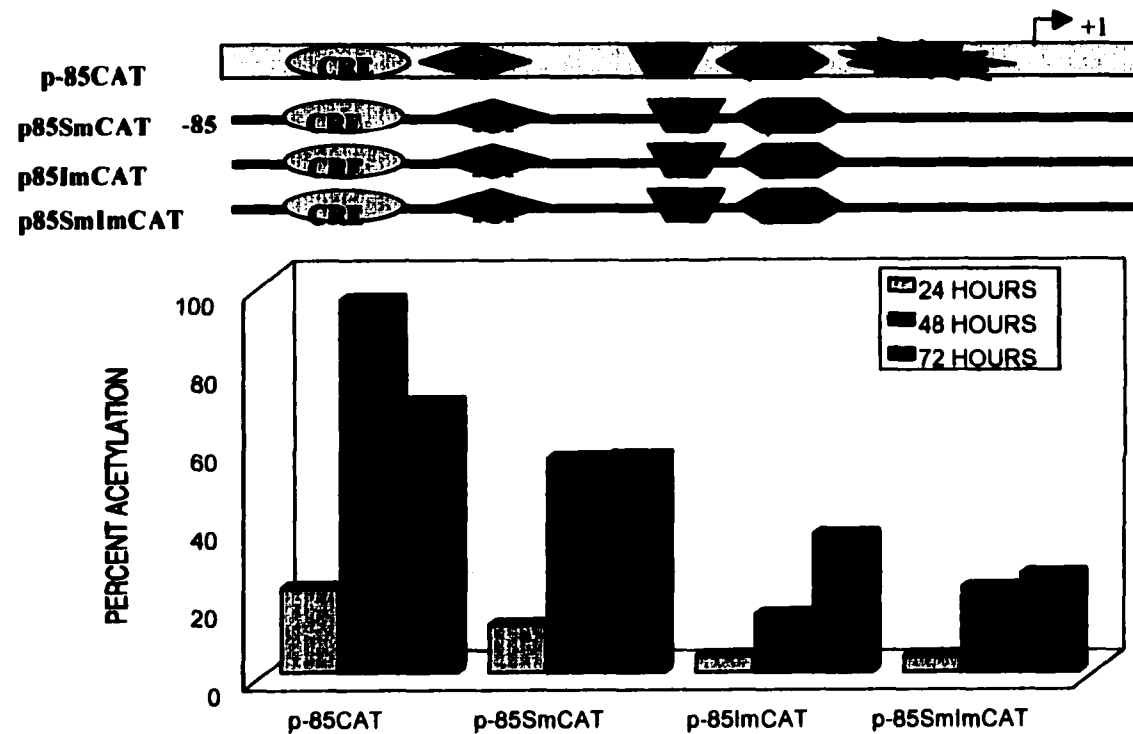


FIG. 18. SIE/ γ IRE mutations in the context of the -85 truncated promoter following superinfection with HCMV. To determine how the mutated elements function in the -85 minimal promoter which has activity at or above wildtype levels, constructs were mutated at SIE, or γ IRE. A double mutation was generated in SIE/ γ IRE as depicted by element(s) shaded in gray. 4 μ g of each mutated CAT-reporter constructs, labelled to the far left, was transfected into human fibroblasts on day one, followed by superinfection with HCMV at 10 pfu per cell on day two. Cell extracts were harvested at 24, 48, and 72 hours and processed to determine UL98 promoter activity. Graphed data is expressed as percent acetylation.

response element (-82 to -75) possesses a regulatory role in UL98 promoter activation, the question arises as to why is it possible to delete the cyclic AMP response element (in the p-64CAT) and maintain a significant level of promoter activity. To address this question, CRE, TCF-1 element, or both were mutated in the context of the -85 and -64 truncated promoters to assess promoter activity in the presence and absence of CRE .

First, the promoter deleted to -85 was mutated at CRE (p85CmCAT), TCF-1 (p85TmCAT) element, SIE (p85SmCAT), or γ IRE (p85ImCAT) (Figures 17, 18).

Double mutations were also generated (CRE/TCF-1 and SIE/ γ IRE). After transfections followed by superinfection with HCMV into HFF1 cells, the cells were again harvested at the respective times (24, 48, and 72 hours postinfection) and assayed for CAT activity. p-85CAT served as a positive control allowing for the functional determination of each element independent of one another in the context of this truncated promoter construct. Figure 17 shows that mutation of the CRE site results in a four fold reduction at 24, 48, and 72 hours. The activity of the promoter mutated at the TCF-1 element was reduced similarly with a slight increase over the CRE mutation in promoter activity early. In the double mutation of CRE and TCF-1 element, promoter activity was reduced even further, indicating that a cooperative effort between CRE and TCF-1 element may take place.

While p85SmCAT had a slight reduction compared to the p-85CAT construct, p85ImCAT was drastically reduced (Figure 18). The decreased activity observed in the SIE/ γ IRE double mutation in the context of the -85 promoter is likely due to the γ IRE mutation since the promoter activity is similar between the p-85ImCAT and the p-85SmImCAT constructs. As observed with the mutations generated in the context of the full-length promoter, CRE and γ IRE are important for UL98 promoter activation.

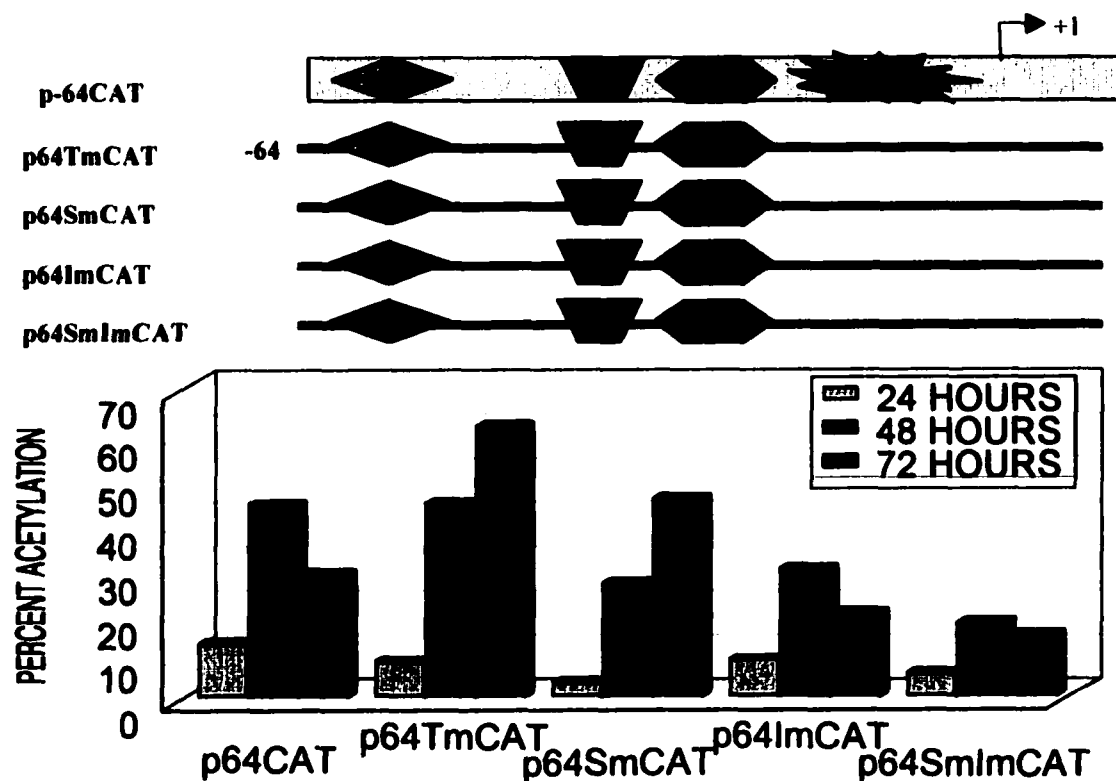


FIG. 19. Assessment of mutated elements in the context of the -64 truncated promoter by transfection/superinfection assay. To study the effect of the TCF-1 element in the presence of the activation sequences, TCF-1 element, SIE, and γ IRE in addition to the double mutation SIE/ γ IRE, were generated and tested in transfection/superinfection assays. The altered promoters (4 μ g) were transfected into fibroblasts followed by an infection with the Towne strain of HCMV at 10 pfu per cell. Cells were harvested at 24, 48, and 72 hours post infection and assayed for promoter activity.

The UL98 promoter deleted to -64 is considered the minimal working promoter because deletions generated to this point have little effect on UL98 promoter activity. To determine how the remaining elements (TCF element, SIE, and γ IRE) function in the absence of CRE, each was mutated in the context of the -64 truncated promoter. Again, all promoter-CAT constructs were tested in superinfection assays. The promoter mutated at the TCF-1 element in the context of the -64 promoter (p64TmCAT) was responsive to viral IE proteins at or above p-64CAT (Figure 19). This observation was unexpected considering a deletion of the TCF-1 element reduced promoter activation to a large extent (See Figure 10). Slightly reduced activity was observed from p64SmCAT at 24 hours postinfection, but this promoter showed activity similar to the wildtype -64 promoter at 48 and 72 hours. p64ImCAT had a two to three fold reduction whether mutated alone or in conjunction with SIE at all timepoints. These data show that a) the UL98 promoter functions in the absence of CRE; b) activation still occurs in the presence of a mutated TCF-1 element; and c) mutation of the γ IRE represses promoter activity thereby showing an impact on UL98 promoter activation.

The possibility of a repressor element downstream of -51 in the deletion mutant p-51CAT was discussed earlier (See Figure 10). An observation in transient transfections in response to IE proteins showed that deletions to -64 resulted in activation, while a deletion to -51 resulted in loss of promoter activity (Figure 10). Further deletion to -34 and -28 at the minimal TATA box resulted in the return of UL98 promoter activation albeit less than the wild-type promoter (Figure 10). Therefore, sequences between -51 and -28 confer a repressed phenotype in the context of the -51 promoter in response to IE expressing plasmids. Sequences between -51 (just downstream of TCF-1

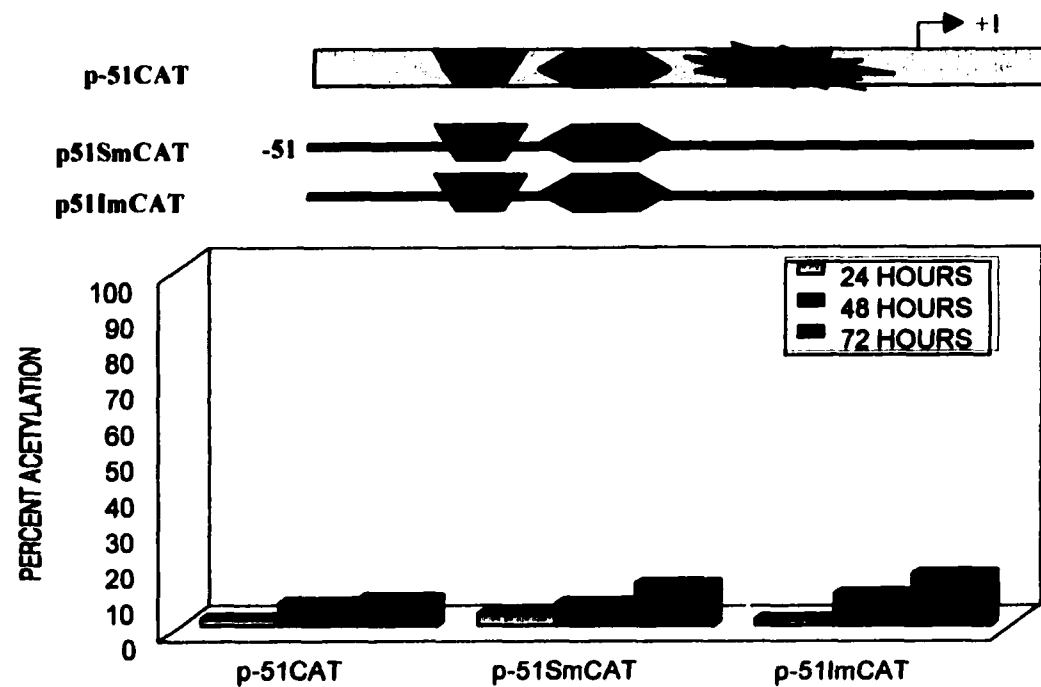


FIG. 20. SIE and γ IRE mutations in the context of the -51 promoter. Mutations within the SIE, γ IRE, or both were generated by overlapping PCR mutagenesis in the -51 truncated promoter. The constructs were transfected into human fibroblast cells followed by superinfection on day two. Again, cells were harvested at 24, 48, and 72 hours. Cell extracts were processed and promoter activity determined by CAT analysis.

element) and -28 (TATA box) include two additional elements: sis/PDGF inducible element (SIE) at -43 and gamma interferon response element (γ -IRE) at -37. The SIE, γ -IRE, and the TATA box are in tandem. To determine whether the SIE and /or γ -IRE act as the repressor sequences, mutations were generated in the SIE and /or γ -IRE, by PCR mutagenesis, in the context of the p-51CAT. Unfortunately, a promoter mutated at both the SIE and γ IRE could not be generated after several attempts. The mutated products were *Hind*III digested, isolated, and cloned into pSVOCATd and tested in superinfection assays. Results from these studies revealed that neither the substitution mutation in SIE nor γ IRE altered the activation state of the promoter deleted to -51 (Figure 20). All three promoters were unable to be activated in response to HCMV infection. This indicates that SIE and γ IRE are not functional in repression, but instead aid in the promoter's ability to activate the UL98 gene. SIE and/or γ IRE may then rely on upstream sequences to participate in the activation of the UL98 promoter. As we were unable to generate the double mutation in the context of -51, the cooperation of SIE and γ IRE could not be addressed in the context of the -51 construct.

Analysis of protein-DNA interaction in UL98 promoter activation

Activation of the UL98 promoter by IE proteins alone or by HCMV infection indicated that there is a cooperative effort among the elements to regulate the UL98 gene. A reduction in activation as a result of a deletion to -51 indicated that the sequences upstream of -51 play an important role. Therefore, for binding studies, a probe that comprises all UL98 promoter elements upstream of -51 was isolated. To assess the binding of mock or HCMV infected cell proteins to the UL98 promoter, a ³²P labeled

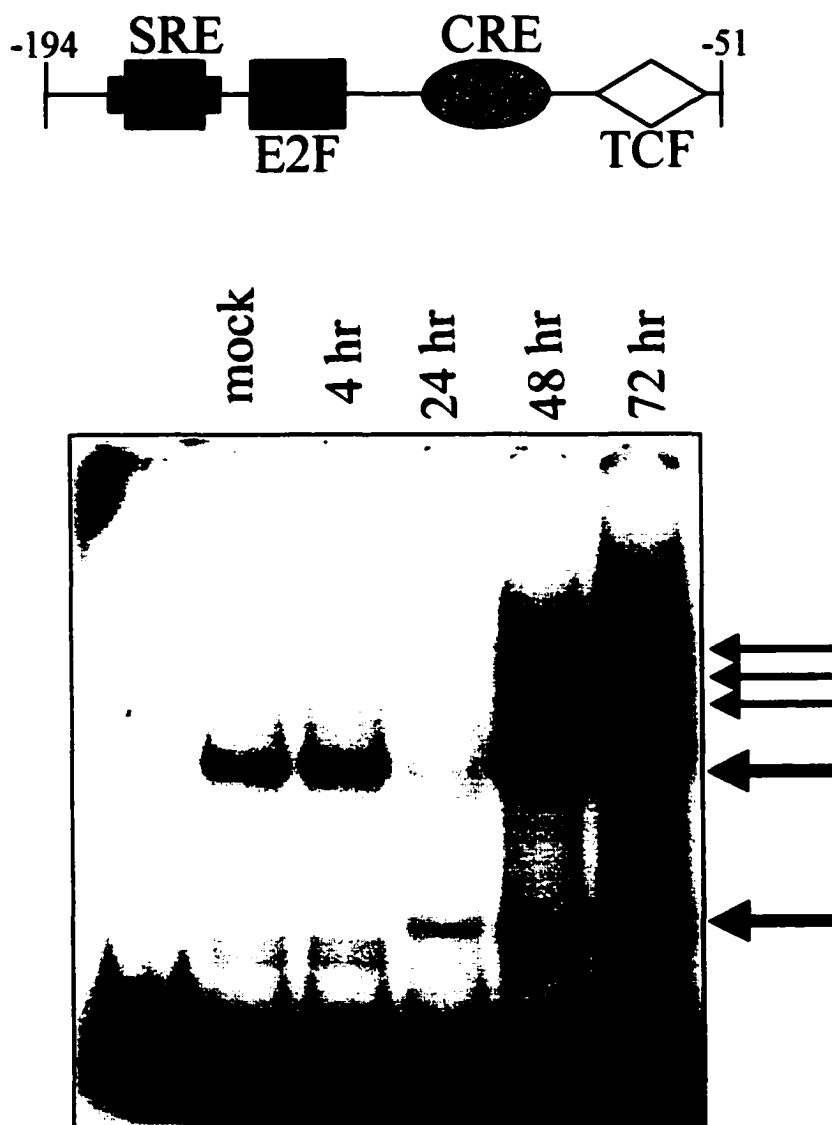


FIG. 21. Binding of infected cell proteins to a UL98 promoter fragment. Nuclear extracts from cells mock infected or infected with HCMV (Towne) were isolated at 4, 24, 48, and 72 hours after infection, lanes 2-6. A ^{32}P radiolabelled UL98 promoter probe (-194 to -51) *NarI* to *MscI* was incubated with nuclear extracts and binding was assessed on a 4% polyacrylamide gel. Lane 1 represents the probe alone. Binding complexes are indicated by arrows.

UL98 promoter fragment of 143 base pairs (bps) (-194 to -51) was incubated with nuclear extracts isolated at 4, 24, 48, and 72 hours after infection (Figure 21). Then, by electrophoresis, the probe/nuclear extract reaction was assayed for binding of mock or infected cell nuclear proteins to UL98 promoter sequences (complexes are designated by arrows). Three complexes formed with mock-infected cell nuclear extracts. Additional complexes were observed that were restricted to HCMV infected cell nuclear extracts at 48 and 72 hours. The 24-hour infected cell extracts only produced a single complex. The 72-hour infected cell nuclear extracts provided several complexes, most of which were retarded above the two predominant bands visualized in mock and /or infected cell extracts and were more abundant. From these data, it was determined that proteins from mock as well as HCMV infected cell nuclear extracts are capable of binding the UL98 promoter (-194 to -51).

Next, a competition-binding assay was used to assess the sequence specificity of the protein-DNA interactions observed in Figure 21. To define the complexes that were UL98 promoter specific, we carried out competition analysis. Mock or infected cell extracts isolated at 72 hours were incubated with 50-fold excess unlabeled probe (lanes 3,6) as competitor prior to the addition of the ^{32}P labeled 143 bp UL98 probe (Figure 22). Analysis of the binding showed that the complexes identified by arrows in mock and upper complexes in infected cell extracts were competed off (lanes 4,7). A nonspecific competitor of 148 bp was isolated from plasmid pSVOCAT. Lacking any UL98 specific sequences, the fragment could not compete off complexes formed in mock or infected nuclear extracts. Results from this figure show that the complexes indicated by the three top arrows are UL98 promoter specific and HCMV



FIG. 22. Competition analysis of the UL98 promoter fragment. Mock (lanes 2-4) or infected (lanes 5-7) cell extracts harvested at 72 hours after infection were incubated with the ^{32}P radiolabeled UL98 promoter probe. Lanes 3 and 6 contain reactions incubated with 100 fold excess unlabeled specific competitor and lanes 4 and 7 contain reaction incubated with 100 fold excess 148 bp nonspecific competitor. Lane 1 represents the probe alone.

CRE/TCF PROBES AND COMPETITORS

<u>CRE</u>	<u>TCF</u>	
5'-CCATGCTGCACGAAT <u>TACGTCAG</u> AAAGAACGTGGAGCG <u>TCTGTT</u> GGCCACGAG-3'		Wildtype
5'-CCATGCTGCACGAAT <u>TACGTCAG</u> AAAGAACGTGGAG-3'		<u>CRE</u>
5'-CCATGCTGCACGAAGAATTCAGAAAGAACGTGGAG-3'		<u>Cm</u>
	5'-AAAGAACGTGGAGCG <u>TCTGTT</u> GGCCACGAG-3'	<u>TCF</u>
	5'-AAAGAACGTGGAGCGGAATTCGGCCACGAG-3'	<u>Tm</u>

FIG. 23. CRE/TCF element probes and competitors generated for gel mobility shift assays. The top line shows the wild-type sequence contained within the UL98 promoter in the 5' to 3' orientation. The elements of interest within this portion of the promoter include the cyclic AMP response element (CRE) and the T-cell specific factor (TCF) element (underlined). The sequence of the elements were altered utilizing synthetic oligonucleotides. The sequence of the wildtype or mutagenic oligonucleotide is indicated by bolded underlined sequence. The names of the wildtype (CRE, TCF) or mutant (CREm, TCFm) oligonucleotides are listed to the far right.

infected cell specific.

The 143 bp UL98 promoter probe (-194 to -51) contained only the first four of six UL98 specific promoter elements. To test all the elements in the UL98 promoter for binding including those downstream of -51, a 239 bp fragment (-194 to +45) was isolated that contains two additional response elements downstream of TCF element: sis-inducible element (SIE) at -43 and gamma interferon response element (γ IRE) at -37. The ^{32}P -labeled fragment of 239 bp was incubated with mock or infected cell nuclear extracts isolated at 72 hours. The protein/DNA complexes formed were present in mock and infected cell nuclear extracts. Also present were two major slow migrating bands (Data not shown). The two top complexes were solely evident in lanes containing HCMV infected cell nuclear extracts isolated at 72 hours. Specific binding for the complexes could be competed off completely using 50 fold excess of an unlabeled 239 bp fragment as specific competitor. Therefore, specific binding was also evident utilizing the 239 bp probe isolated from the UL98 promoter.

In the next series of experiments synthetic oligonucleotides containing specific UL98 promoter response elements were used in the place of isolated DNA fragment (Figure 23). These elements were found important in transient assays and were examined in binding studies. To assess the binding of proteins to the transcription factor binding site CRE, a ^{32}P labeled oligonucleotide of 36 bp containing only CRE was incubated with mock or infected cell extracts harvested at 72-hours. A single dominant complex was present in mock (depicted by the bottom arrow), while three additional complexes were identified in infected nuclear extracts (identified by arrows) (Figure 24). Competition analysis using 50x and 100x excess unlabeled competitor showed that the top complex

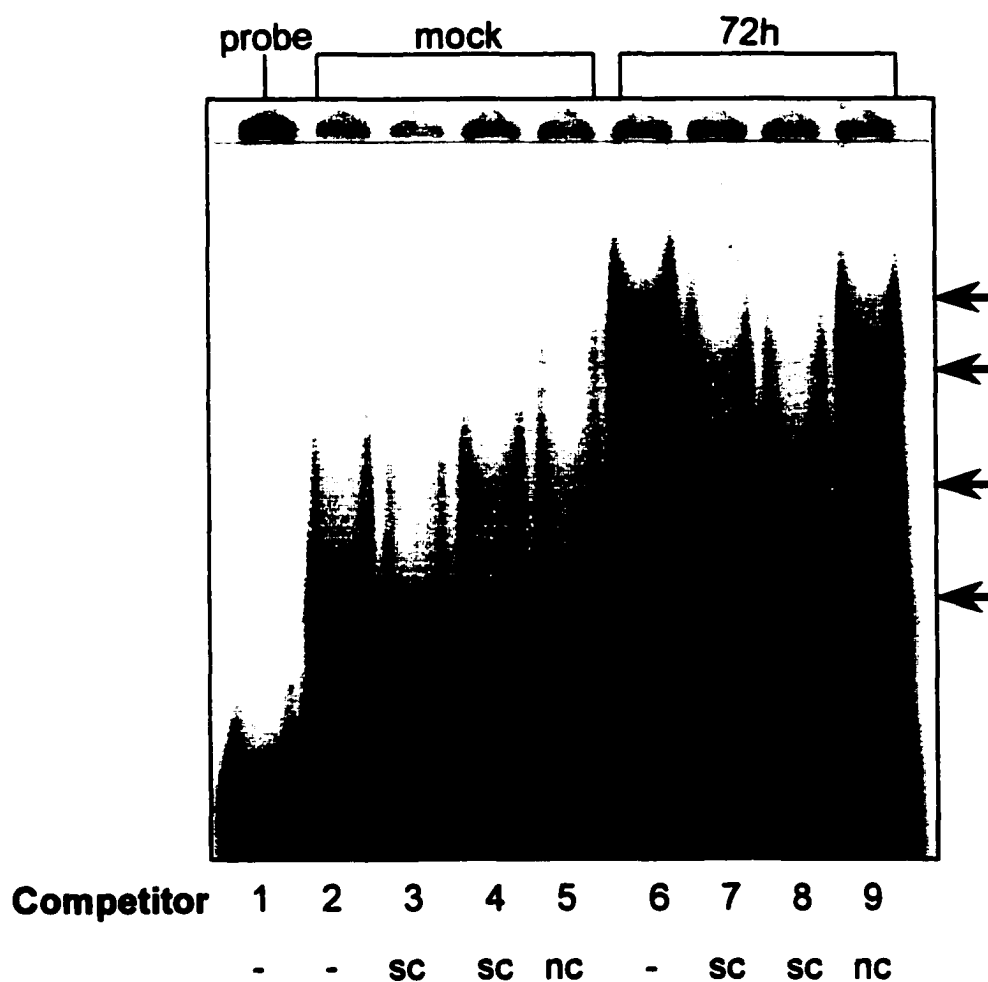
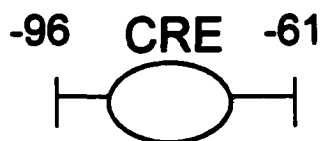


FIG. 24. Binding and competition of the UL98 CRE sequence. A ^{32}P radiolabeled 36 bp CRE oligonucleotide probe was incubated with mock (lanes 2-5) and infected (lanes 6-9) nuclear extracts harvested at 72 hours post infection. Reactions were incubated in the absence (lanes 2 and 6) or presence (lanes 3 and 7/ lanes 4 and 8) of 50 fold and 100 fold excess unlabeled specific competitor (sc) respectively. Lanes 5 and 9 contain 100 fold excess unlabeled nonspecific competitor (nc) of similar size (42 bp). Lane 1 consists of probe only. Binding complexes are indicated by arrows.

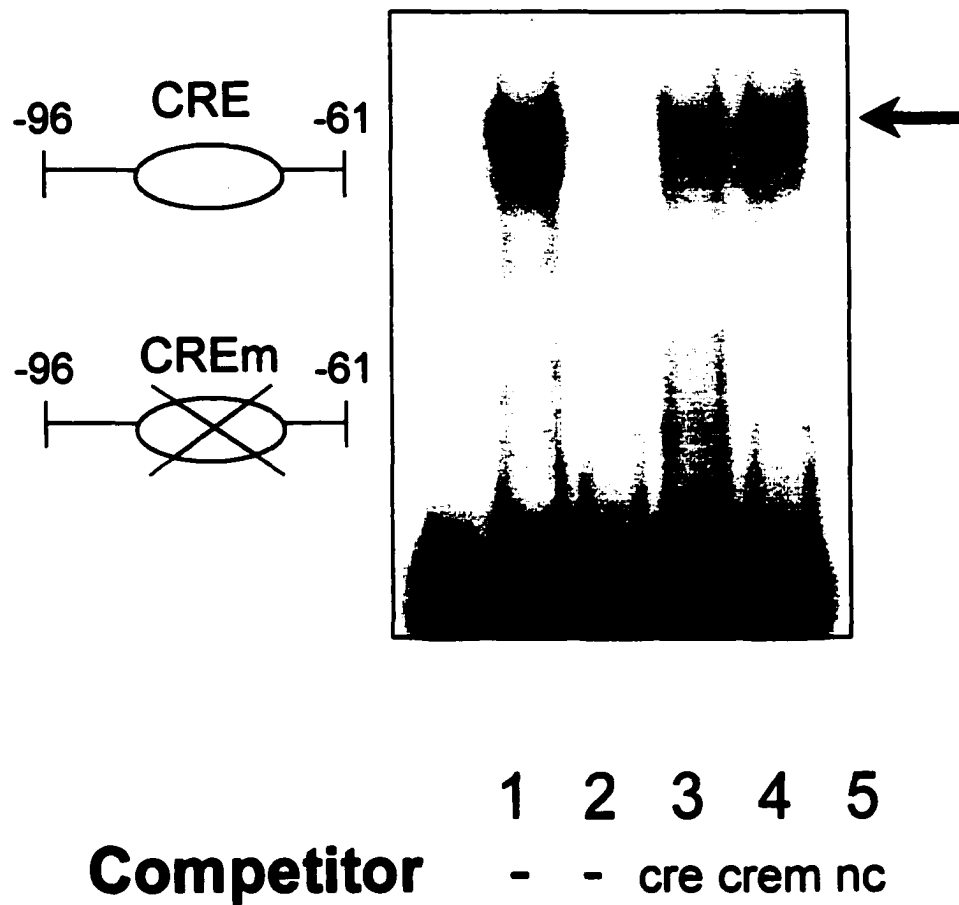


FIG. 25. Mutant CRE probe (CREm) in competition assays. 100 fold excess of unlabeled competitor in lane 3 (specific), lane 4 (mutant), and lane 5 (nonspecific) were incubated with nuclear extracts harvested at 72 hours postinfection. The 36 bp ^{32}P radiolabeled probe (CRE) was then added. Lane 1 represents probe alone and lane 2 contains no competitor.

was specific and could be competed off (Figure 24, lanes 7 and 8). A nonspecific competitor of 42 bp could not disrupt the complex (Figure 24, lane 9). These data show that the complex containing CRE is specific for a protein(s) in 72-hour infected cell nuclear extracts.

In Figures 21 and 22, specific binding was shown by competition analysis. A more definitive way to determine protein-specific binding is to use a fragment identical to the probe fragment except mutated in the binding site thereby disrupting function and presumably binding. To further confirm CRE specific binding, a 36 bp oligonucleotide mutated at CRE (CREm; see figure 23 for sequence) was generated and used as a specific competitor. The oligonucleotide was incubated with HCMV infected cell nuclear extracts isolated at 72-hours followed by the addition of a ^{32}P radiolabeled CRE probe also of 36 bp. This would determine if the specific competition observed in Figure 24 was due to the presence of a CRE site. The results showed that while the 36 bp competitor containing an intact CRE competes off the CRE specific complex (Figure 25 lane 3), neither a 100 fold excess cold CREm (lane 4) nor a 42 bp nonspecific competitor (lane 5) could compete off the CRE specific complex. The observations in Figure 25 show that an intact CRE must be present for the binding of proteins from 72-hour HCMV infected cell nuclear extracts to the 36 bp CRE probe.

Antibody supershift assays are used to identify proteins present in a protein/DNA complex. To determine the HCMV infected cell protein(s) that bind to the CRE site in the UL98 promoter, supershift analyses using CREB/ATF antibodies were carried out. The ^{32}P radiolabeled CRE probe was incubated with 72-hour nuclear extracts (Figure 26, lane 2), followed by the addition of CREB, ATF-1, or ATF-2 antibody (lanes

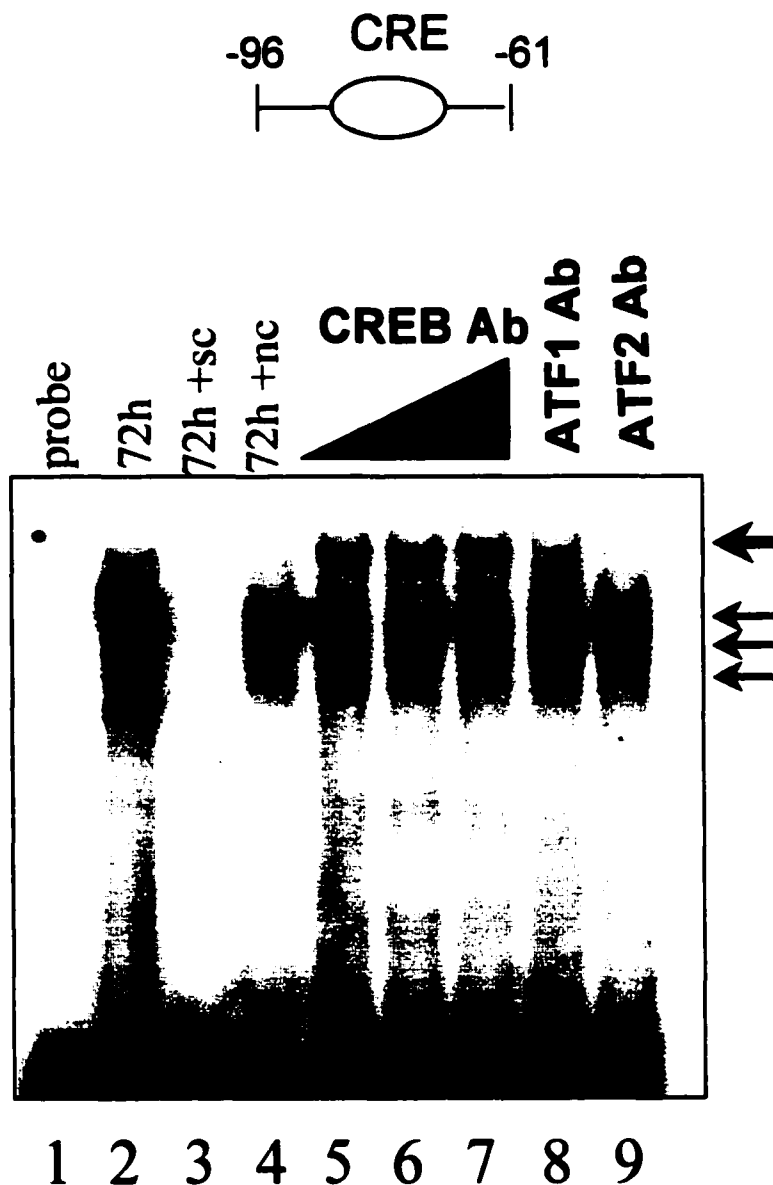


FIG. 26. Identification of protein binding the UL98 promoter. A ^{32}P radiolabeled 36 bp probe, shown to the left, was incubated with nuclear extracts and further incubated without (lanes 1-4) or with (lanes 5-9) specific antibodies for the CREB/ATF protein family. Lanes 5-7 contain reactions with increasing amounts of the CREB antibody, and lanes 8 and 9 contain ATF 1 and 2, respectively. Lane 1 shows probe alone.

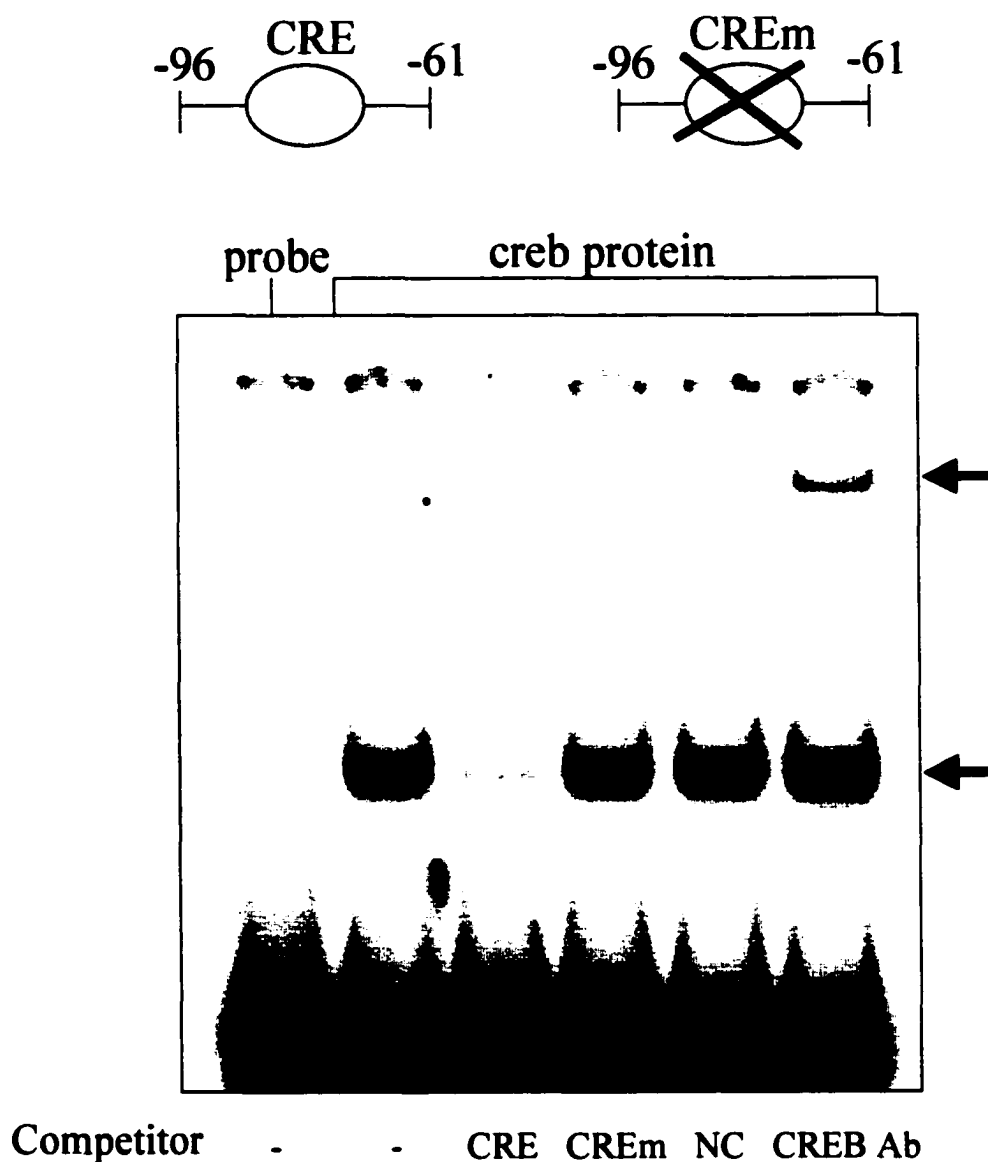


FIG. 27. Confirmation of cyclic AMP response element as a binding protein of the UL98 promoter. A ^{32}P labeled CRE probe of 36 bp was incubated with diluted CREB protein (Santa Cruz) at 100 $\mu\text{g}/\text{ml}$ and was diluted in buffer D at a ratio of 1:100 (lanes 2-6). Lane 1 represents probe only. The complex was competed with the 100 fold excess of unlabeled CRE probe (lane 3), mutated CRE (lane 4), and a nonspecific competitor (NC) of 34 bp (lane 5). Lane 6 contains CREB antibody for supershift analysis.

5-9). While the addition of CREB antibody in increasing quantity resulted in a supershift of the CRE specific complex (lanes 5-7), ATF1 antibody (lane 8) or ATF2 antibody (lane 9) addition did not result in a supershift, suggesting that one of the proteins binding to CRE is CREB protein (Figure 26). Although the ATF-1 antibody failed to show a supershift upon addition (lane 8), its presence within the reaction noticeably changes the binding profile.

To provide further evidence that the protein binding the CRE sequence was CREB, a ^{32}P labeled CRE oligonucleotide probe of 36 bp was incubated with serial dilutions of CREB protein (data not shown). A 1: 100 dilution of CREB protein in buffer D efficiently showed binding of CREB protein to the CRE sequence (Figure 27, lane 2). Competition analysis using excess cold CRE oligonucleotide as a competitor confirmed that CREB is binding CRE as binding was disrupted (Figure 27, lane 3). A mutated CRE or a nonspecific competitor (Figure 27, lane 4 and 5), could not compete off the complex. The addition of CREB antibody (Figure 27, lane 6) resulted in a supershift of the CRE specific complex. These data does in fact show that CREB protein binds the CRE site within the UL98 promoter.

The element for TCF-1 may play a role in UL98 promoter activation as suggested by data in deletion analysis (see Figure 10 and 11). A probe containing the TCF element was used in gel shift assays to assess the binding of HCMV viral or virally induced proteins to the TCF sequences. A ^{32}P labeled TCF probe (-74 to -45) of 29 bp was incubated with 72 hour mock or infected cell nuclear extracts (Figure 28). Several complexes were formed; however, four complexes in particular were specific for infected cell nuclear extracts (lanes 6-9; indicated by arrows). Competition assays using 100 fold

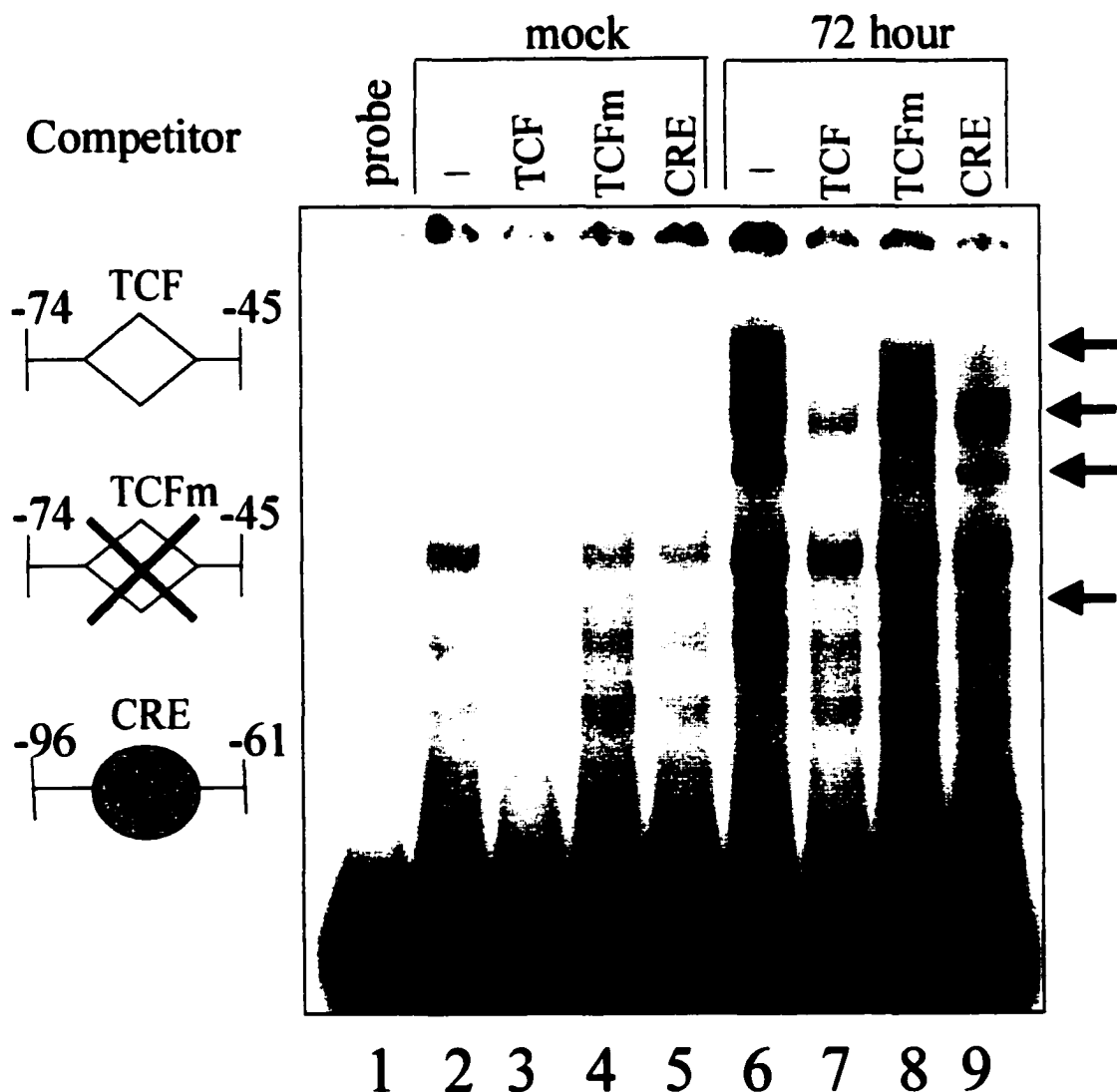


FIG. 28. Binding and competition of the UL98 TCF sequence. A ^{32}P labeled 29 bp TCF oligonucleotide probe was incubated with mock (lanes 2-5) and infected (lanes 6-9) nuclear extracts each harvested at 72 hours post infection. In competition assays, reactions were incubated in the absence (lanes 2,6) or presence of competitors for TCF (lanes 3,7), mutant TCF (lanes 4,8), or CRE (lanes 5,9). Reactions were run through a 6% polyacrylamide gel. Lane 1 represents probe alone.

SIE/ γ IRE MUTANT PROBES AND COMPETITORS

<u>SIE</u> <u>γIRE</u>	
5'-GGCCACGAGC <u>GACGGG</u> CTGTATTATATAA-3'	Wildtype
5'-GGCCACGAGC <u>GAATTC</u> CTGTATTATATAA-3'	<u>Sm</u>
5'-CCACGAGCGACGGG <u>GAATTC</u> TTATATAA...CAGC-3'	<u>Im</u>
5'-CCACGAGC <u>GAATTC</u> GAATTCCTTATATAA...TTCG-3'	<u>SmIm</u>
5'-GCGTCTGTTGG <u>GAATTC</u> GC <u>GAATTC</u> GAATTCCTTAT-3'	<u>USI3m</u>

FIG. 29. SIE/ γ IRE probes and competitors generated for gel mobility shift assays. The top line shows a portion of the wild-type sequence containing the sis-inducible element (SIE) and the gamma interferon response element (γ IRE). Each cis-acting element is bolded with a line below SIE and a line above γ IRE to denote the beginning and end of each element. Below, four oligonucleotides are represented 5' to 3' and bolded letters indicate a mutation from wild-type sequence to an *EcoRI* site. The site(s) mutated is underlined to the far right. Sm-mutated SIE, Im-mutated gamma IRE, SmIm-mutated at SIE and gamma IRE, and USI3m-triple mutation upstream (U) of SIE, at SIE, and at gamma IRE.

excess TCF unlabeled probe resulted in the loss of three of the four complexes (lane 6; two complexes to a lesser extent). More interestingly, the addition of 100 fold excess cold CRE competitor resulted in a loss of two complexes (lane 9; the two middle complexes to a lesser extent). Both the top and bottom complex were competed off by the CRE competitor and the TCF-1 competitor. A mutant TCF (TCFm; see figure 23 for sequence) oligo could not completely compete off any of the complexes. These data show that in some instances, each protein, CREB and TCF-1 or a TCF-1 related protein, may bind the TCF-1 element probe simultaneously or each may bind the promoter sequences separately. Studies by others have shown that TCF-1 is made up of four different isoforms for different regulatory controls (204). Binding of specific isoforms may be represented in Figure 28. Supershift assays using TCF-1 antibody are necessary to determine the binding of TCF-1 to CRE and /or TCF-1 element. However, TCF-1 antibody is not yet available to address actual TCF-1 protein binding.

Assessment of promoter activity from transient transfection data indicated that the γ IRE is functional in promoter activation in the context of the full length promoter (pUL98CAT). The next aim is to utilize gel mobility shift assays to assess binding of mock or HCMV infected cell nuclear extracts to the SIE and γ IRE sequences which are in tandem. Sequences used in the following experiments are represented in Figure 29 as synthetic oligonucleotides 5' to 3'. To first assess the protein/DNA interactions that occur at the SIE/ γ IRE, a 30 bp oligonucleotide end labeled with ^{32}P was incubated with mock or HCMV infected cell nuclear extracts both isolated at 72-hours (Figure 30). Both mock and infected cell extracts showed very similar binding profiles: one large broad

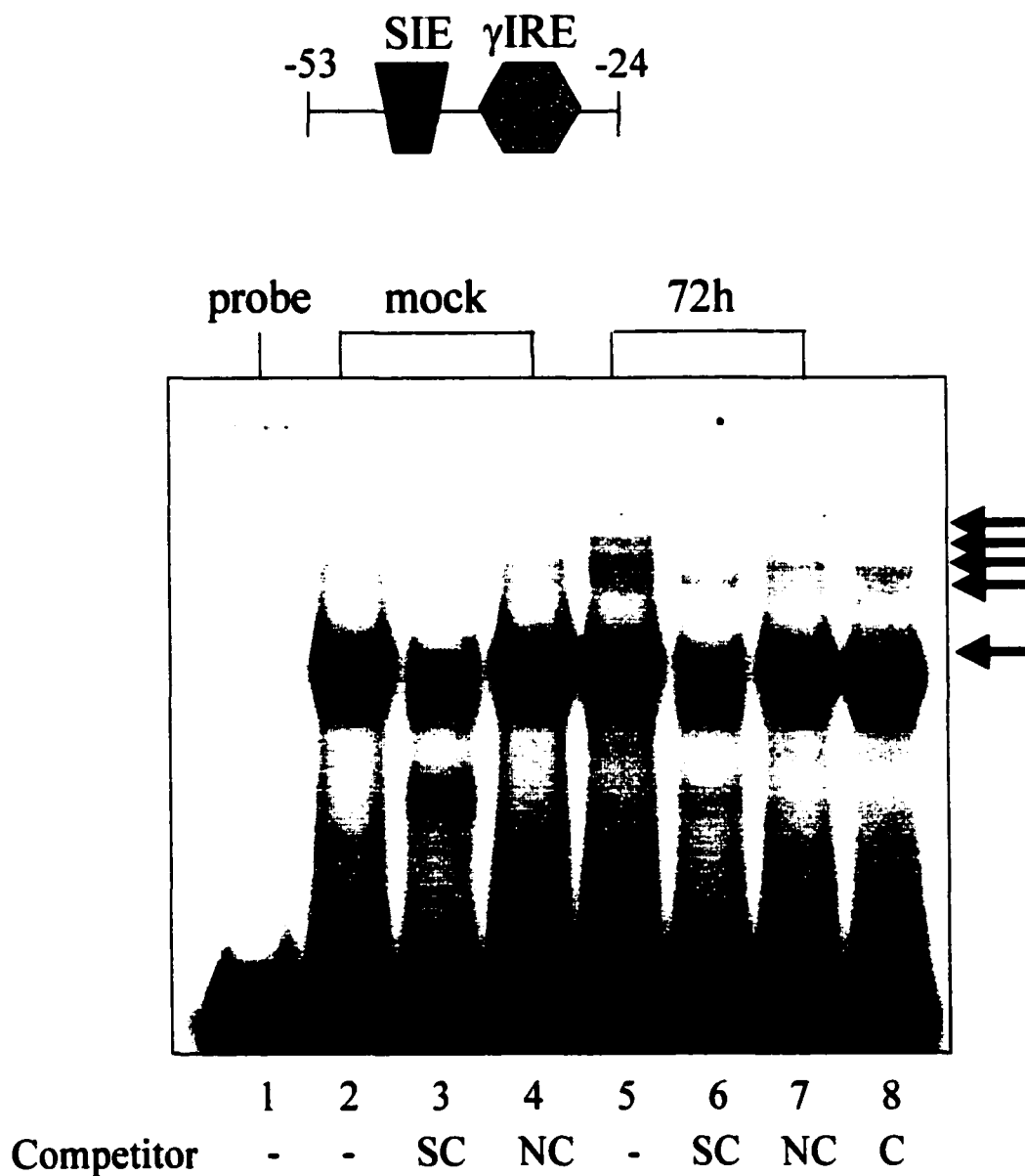


FIG. 30. Binding and competition of the UL98 SIE and γ IRE sequences. A ^{32}P radiolabeled 30 base pair (bp) oligonucleotide probe was incubated with mock (lanes 2-4) and infected (lanes 5-7) nuclear extracts harvested at 72 hours post infection. Reactions were incubated in the absence (lanes 2 and 5) and presence (lanes 3 and 6) of 50 fold excess unlabeled specific competitor (SC). Lanes 4 and 7 contain 50 fold excess unlabeled nonspecific competitor (NC) of similar size (34 bp). Lane 8 contains a control (C) 72 hour extract and lane 1 consists of probe only. Complexes are indicated by arrows.

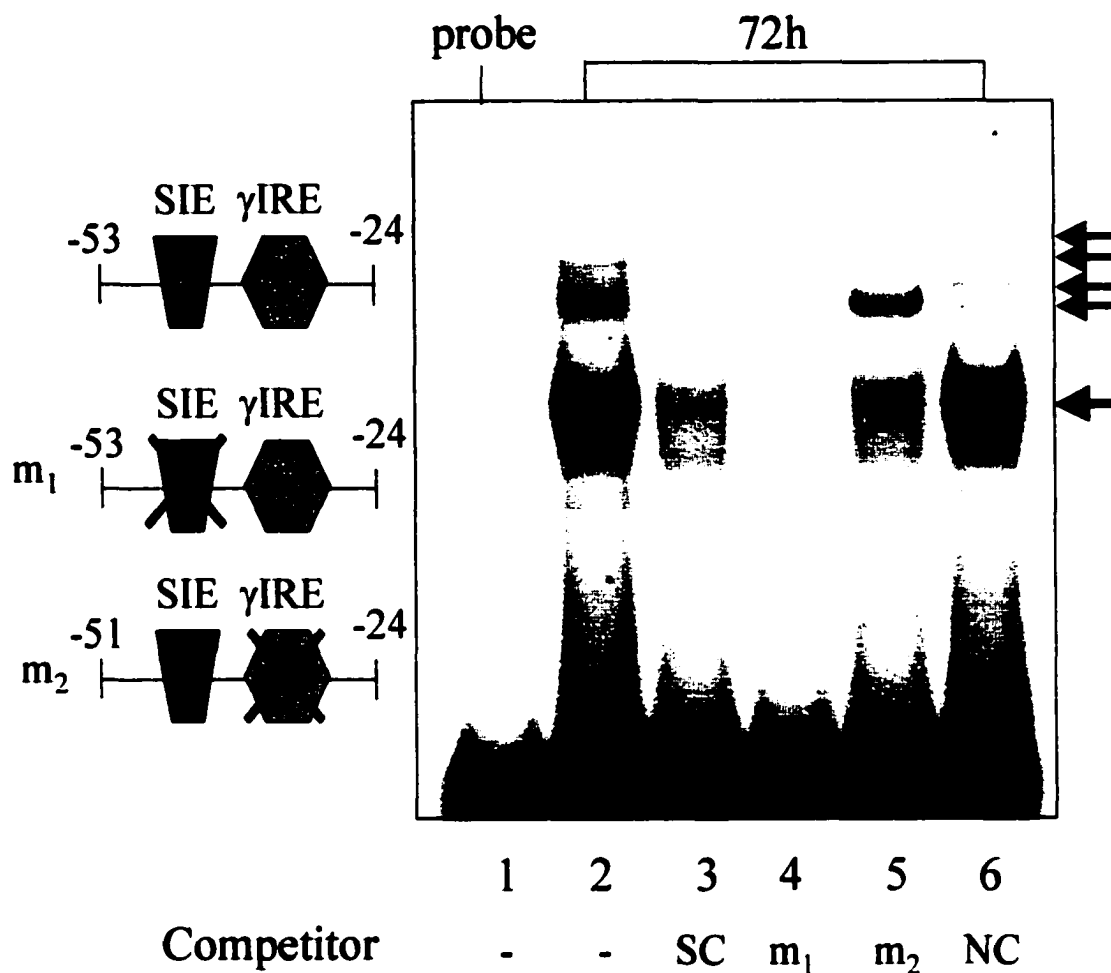


FIG. 31. Competition analysis by gel mobility shift assay utilizing mutant probes. A ^{32}P 30 bp oligonucleotide was incubated with 72 hour extracts were preincubated with 100 fold excess unlabeled competitors: wild-type SIE/ γ IRE (lane 3), or m_1 -mutated SIE (lane 4), or m_2 - mutated γ IRE (lane 5). Lane 6 contains a nonspecific competitor and lane 1 represents probe alone.

band, which may be several bands migrating close together at midpoint in the gel, as well as several lighter bands migrating above (Figure 30, lanes 2,5). Those minor bands present in mock extracts seem to be upregulated in 72 hour infected cell extracts. A 50 fold excess of a 30 bp cold (unlabeled) specific competitor was preincubated with the nuclear extracts prior to the addition of the 30 bp labeled probe. While the broad band could be competed off although not completely, the bands migrating more slowly (top) were competed off well (Figure 30, lanes 3, 6). A nonspecific competitor of 34 bp (Figure 30, lanes 4, 7) could not disrupt the bands observed in Figure 30, lanes 2, 5. Lane 8 is a control (C) extract incubated with the 30 bp SIE/ γ IRE probe. From these data, it was observed that the three lighter bands, which form above the broad band, is specific for the SIE/ γ IRE probe and appeared only in the lanes containing HCMV infected nuclear extracts isolated at 72 hours.

To determine the importance of the sequences within the 30 bp SIE/ γ IRE probe, another 30 bp oligonucleotide mutated at the SIE and a 50 bp oligonucleotide mutated at the γ IRE were used in competition assays as competitors (Figure 31). Competitors (m_1 - m_4 ; see figure 29 for sequences) were preincubated with 72 hour infected cell nuclear extracts followed by incubation with the 30 bp SIE/ γ IRE 32 P labeled probe. Results in Figure 31 show that a mutated SIE/intact γ IRE (m_1) competes for binding of specific complexes more effectively than the competitor containing the intact SIE/mutated γ IRE (m_2) although both compete well under these conditions. It appears that the data, observed in this assay, shows that the γ IRE may bind a specific regulatory protein(s), which falls in line with the role of γ IRE in the participation of UL98 promoter regulation. SIE binding may be important as well and its potential role should not be overlooked. A

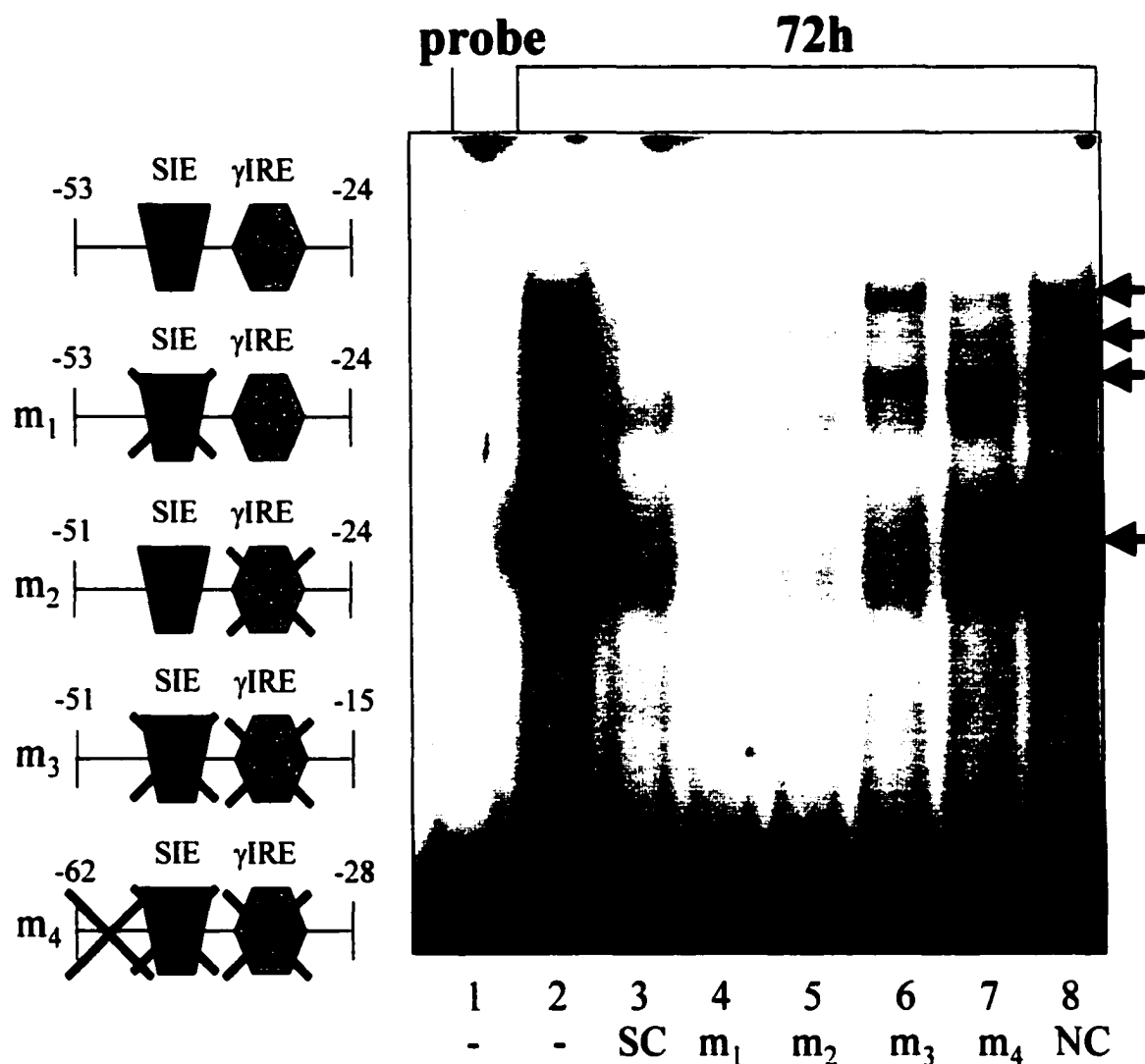


FIG. 32. Competition analysis by gel mobility shift assay utilizing double and triple mutant probes. A ^{32}P 30 bp oligonucleotide incubated with 72 hour extracts was first preincubated with 100 fold excess unlabeled competitors: wild-type SIE/ γ IRE (lane 3), m₁-mutated SIE (lane 4), m₂- mutated γ IRE (lane 5), m₃-doubly mutated SIE/ γ IRE (lane 6), m₄-triple mutated upstream of and at SIE/ γ IRE (lane 7). Lane 8 contains a nonspecific competitor and lane 1 represents probe alone.

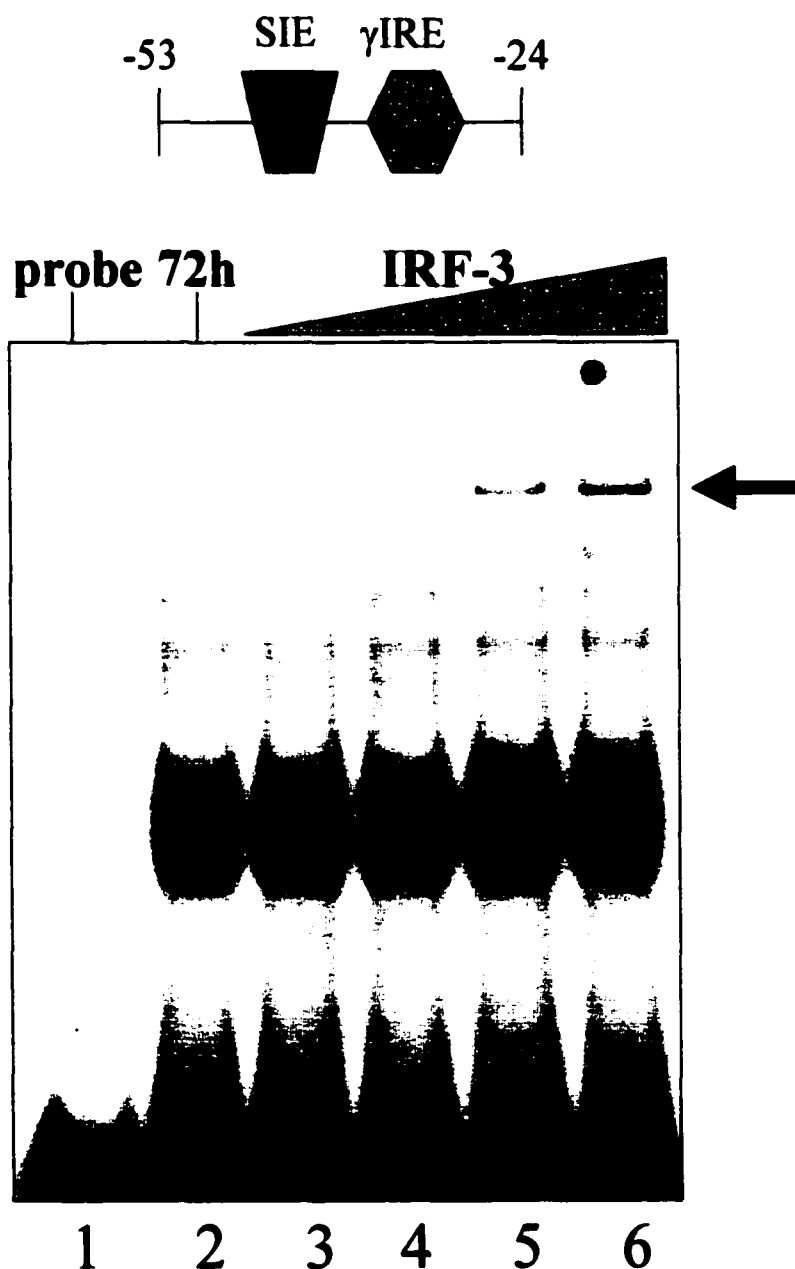


FIG. 33. Supershift assays utilizing IRF-3 antibody. A ^{32}P radiolabeled 30 bp probe was incubated initially with 72 hour HCMV infected cell nuclear extracts (lane 2). Following a 15-20 minute incubation, increasing amounts of anti-IRF-3 antibody were added to the reaction at 0.5, 1.0, 2.0, and 4.0 μl (lanes 3-6). Lane 1 contains probe alone.

37 bp oligonucleotide doubly mutated at the SIE and the γ IRE (m_3), as well as a 35 bp oligonucleotide containing three mutations (upstream of SIE, SIE, and γ IRE) (m_4) were also used as competitors in Figure 32. All competitors could compete with varying effectiveness. Still again, the 30 bp oligonucleotide containing a mutated SIE and an intact γ IRE competed best (Figure 31). Therefore, the γ IRE is important in protein/DNA interactions involving the UL98 promoter with HCMV infected cell nuclear proteins.

Up to this point, a basis for specific SIE/ γ IRE binding had been established. Activation of the UL98 promoter appears to preferentially rely upon γ IRE binding rather than SIE binding (Figure 32). However, due to the tandem nature of the SIE/ γ IRE sequences, a role for SIE binding cannot be ruled out. Various interferon regulatory factors (IRF) and signal transducers and activators of transcription (STAT) proteins play an important role in the activation of interferon (IFN) inducible genes which bind γ -IFN induced elements (160, 207, 228). Recent studies provided evidence for the IRF-3 and CBP proteins cooperating in the regulation of an early promoter through binding an interferon stimulated response element (ISRE). To assess whether IRF-3 binds UL98 SIE/ γ IRE sequences, an anti-IRF-3 antibody was acquired and used for supershift analysis following the incubation of the 32 P radiolabeled SIE/ γ IRE probe with HCMV infected cell nuclear extracts isolated at 72 hours. The anti-IRF-3 antibody was administered in increasing amounts and found to supershift the SIE/ γ IRE/ protein binding observed in Figure 32 (Figure 33, lanes 3-6). This was in contrast to the 72 hour infected nuclear extract lacking antibody (Figure 33, lane 2). These data suggested that IRF-3 is a component of the proteins that participate in the regulation of the UL98 promoter.

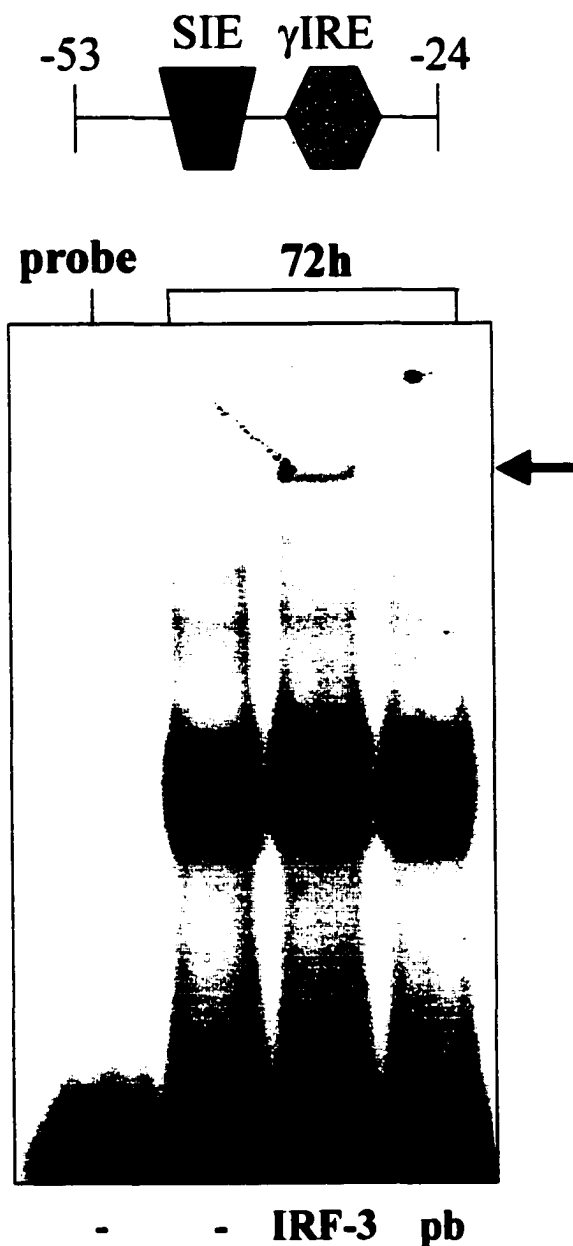


FIG. 34. Confirmation of specific IRF-3 binding. Following the incubation of a ^{32}P SIE/ γ IRE 30 bp probe with HCMV 72 hour infected cell nuclear extracts (lane 2), 4.0 μl of IRF-3 antibody was added (lane 3). A nonspecific antiserum (prebleed) was used (lane labeled pb) to examine the specificity of the anti-IRF-3 antibody. Lane 1 is probe alone.

To confirm the specificity of the IRF-3 antibody supershift, a rabbit antiserum was used in direct comparison to anti-IRF-3 in a supershift experiment following incubation of 30 bp SIE/ γ IRE probe with 72 hour HCMV infected cell nuclear extract. Unfortunately, as shown in Figure 34, the nonspecific antiserum (rabbit prebleed) supershifted just as the anti-IRF-3. These data suggests that the immune reaction was not specific. IRF-3 as a component of the trans-acting factors for UL98 promoter activation could not be proved conclusively from these data. Further experiments using IRF-3 protein in the presence of the SIE/ γ IRE 30 bp probe could provide evidence for IRF-3 as a UL98 promoter activation component.

CHAPTER V

DISCUSSION

Activation of the HCMV UL98 Early Promoter

The goals of the project were to isolate the 5' regulatory sequences of the HCMV UL98 promoter which was isolated as a 421 bp fragment (-309 to +112), and to characterize its activity in the presence of the immediate early transactivators or virus. This approach would allow for the confirmation of the UL98 promoter as an early promoter and determine the IE requirement for activation (1). Once the UL98 promoter sequences were isolated, the sequences were cloned upstream of a reporter gene chloramphenicol acetyltransferase. As presented in figure 3, the promoter was strongly activated in the presence of the effectors IE72 and IE86 expressed from the plasmid pSVH. This was demonstrated by cotransfection of the plasmid pUL98CAT with pSVH (1). Previously, the UL54 polymerase (pol) promoter was characterized and found to require IE gene products for its activation (reviewed in reference 186). The promoter for the polymerase gene acted as a control for promoter expression. The IE requirement was controlled for by the use of the expression plasmid derived from simian virus (SV) 40, which lacks the exons for IE1 and IE2 leaving intact the vector backbone called pSVOd. Although the UL54 and UL98 genes are expressed at early times, each belongs to a different early subclass. While the pol gene is expressed at approximately the same levels throughout the course of infection, the UL98 alkaline nuclease gene is expressed at low levels (RNA) early and continually increases through late times. The latter expression pattern was observed in two other early genes; the pp65 gene and the 1.2 kb RNA gene (38, 208). The IE transactivator requirement for the pp65 promoter utilized

both IE72 and IE86. The 1.2 kb RNA promoter only required the IE86 protein for maximal activation. By testing the UL98 promoter in the presence of each IE transactivator separately and within the same plasmid, the IE requirement for UL98 promoter activation was revealed. Both IE72 and IE86 were necessary for maximal UL98 promoter activation (Figure 4). The genomic construct, pSVH, activated the UL98 promoter most efficiently. pSVH expresses the minor as well as the major IE proteins from the MIE gene region suggesting that the MIE minor products likely enhance activation of the UL98 promoter.

The HCMV IE72 and IE86 regulatory proteins have been implicated in early promoter activation (38, 94). How these viral transcription factors exercise their role in the activation of early viral promoters has not yet been revealed. Protein-protein interaction has been observed for both IE72 (61, 108, 113) and IE86 (18, 82, 103, 163, 165). IE86 is a more promiscuous transactivator interacting with cellular transcription factors (CREB, c-jun, ATF-2, jun-B, NFkB, p300/CBP, SP-1, TEF-1, and UBF) as well as components of the basal transcription machinery (TFIIB, TBP). IE86 binds to its own promoter at a cis repression signal (CRS) of 10 AT rich nucleotides between two CG residues (CG-N₁₀-CG) and represses transcription. The phosphoprotein also binds specific sequences located upstream of the TATAA box of HCMV early promoters (164, 165, 180, 182). The sequences within early promoters resemble the CRS of the MIE promoter. However, the CG residues are at both ends of a 14 nucleotide sequence that is AT rich internally (180).

The UL98 promoter has a CG-N₁₄-CG sequence from -102 to -85 positioned adjacent to the CRE site from -82 to -75. Also overlapping the CRE and the TCF-1 like

element are two CG-N₁₆-CG sequences (-85 to -67, -67 to -47). The nucleotides sandwiched between the CG residues ranging from 9 to 14 residues fits well with the thought that IE86 may recognize structural features of the DNA more than specific sequences (180). Because IE86 is known to interact with the basal transcription machinery as well as with CREB, perhaps IE86 links CREB and TBP or TFIIB for transcription initiation. IE86 could interact with CREB protein that binds CRE or an IE86 binding site within the UL98 promoter. IE86/CREB interaction could be tested indirectly by assessing the ability of IE86 antibody to supershift the CREB/UL98 specific CRE complex in gel mobility shift analysis. This will suggest that IE86 is a part of the complex binding CRE. Competition experiments could test the actual binding of IE86 to CREB. Bands retarded by a CREB/ CRE complex could be enhanced or stabilized by the addition of purified IE86. Again, anti-IE86 could be added to further retard the complex indicative of IE86/ CREB interaction. Similar experiments using UL98 promoter specific IE86 binding sites as the DNA probe could also be employed. Neither has been demonstrated thus far. This may provide some indication as to why removal of sequences beyond -64 results in the decrease of UL98 promoter activation.

The subclassification of early genes is based upon pattern of expression throughout the course of infection. Because the RNA levels of the UL98 gene increased throughout the course of infection in northern blot analysis (1), promoter activation was also assessed via analysis of CAT activity at 24, 48, and 72 hours postinfection. The UL98 promoter was transfected into cells followed by HCMV infection. Transient transfection data showed that the UL98 promoter was additionally stimulated well above activation levels observed in the presence of viral MIE proteins (24 hours) and increased

through late times (48 and 72 hours) (see figure 11).

Over the course of infection, UL98 promoter activation increased at 24, 48 and 72 hours (see figure 15 and 16; pUL98CAT). These data were expected considering that previous studies showed that the RNA levels increased in a similar manner (1). However, in figure 11, activation of the UL98 promoter seems to peak at 48 hours and decrease slightly at 72 hours. The transcriptional regulation of the UL98 promoter was assessed by determining the transcriptional initiation of the chloramphenicol acetyltransferase (CAT) gene under the control of the UL98 5' regulatory sequences. The expression of the CAT gene is monitored by measuring the enzymatic activity of the CAT protein. By CAT analysis, it is possible to assess the promoter's ability to mimic accurately the expression pattern of the endogenous gene (UL98 gene).

While there are several advantages for assessing promoter regulation indirectly using a reporter assay, a few limitations should be noted. In transient transfection assays, plasmids exist in an artificial configuration and the copy number may lead to inactivity or aberrant function of specific control elements. The plasmid is also episomal and not in the proper chromosomal configuration. The promoter may function in an aberrant manner. Another disadvantage is the stability of the CAT mRNA. The CAT protein is considered quite stable throughout the course of the reporter assay with a half-life of approximately 50 hours in mammalian cells (9, 111). However, CAT mRNA is present at low levels and is very difficult to analyze directly. If CAT message cannot be detected, there is difficulty in stating whether the level of CAT enzyme appropriately reflects the initiated message from the promoter. The stability of the mRNA was not accounted for in these analyses. To accurately assess transcription in the artificial context

of the transient transfection assay would require the use of a procedure that will measure transcription initiation from the endogenous gene; the nuclear run on assay. This assay would provide a measure of the frequency of transcription initiation independent of the effects of mRNA stability.

Deletion Analysis of the UL98 Promoter

The next question addressed in this study was to determine which response elements upstream of the CAP site were important in UL98 promoter activation (1, 223). The question was approached by deleting one element at a time beginning at the 5' end and in a stepwise manner progressively shortening the promoter to the TATA box. As compared to the wildtype promoter only one of nine deletions drastically reduced promoter activity. A deletion from -64 to -51 reduced promoter activity by more than 70% in the presence of HCMV IE regulatory proteins (Figure 10). Sequences between -64 and -51 have a putative T-cell specific factor (TCF-1) element on the negative strand suggesting this transcription factor binding site may play a significant part in the activation of the UL98 promoter. In the presence of the virus (HCMV), the identical -51 deletion drastically reduced promoter activation by as much as 80% at all time points (24, 48, and 72 hours) (Figure 11). Promoters deleted to -34 and -28 were also reduced significantly.

The TCF-1 binding site is homologous to the LEF-1 site (TCF1 α) found in the TCR α chain gene promoter (20, 202). The LEF-1 (TCF-1 α) protein has high homology to the TCF-1 protein (approximately 97%) and each contains a DNA binding sequence referred to as the HMG domain (202). In other promoters, the ability of a TCF-1 binding

site to support transactivation is context specific (49, 205). Full activation of a promoter with a TCF-1 site depends heavily on the simultaneous presence of other sites (ATF/CREB, core-binding factor/PEBP2 α , and ETS-1) at defined flanking positions (49). The TCF-1 protein has been shown to act not as a transactivator, but rather a facilitator of DNA bending allowing widely separate sites to be brought into close proximity for the activation of the promoter (49). In the UL98 promoter, the TCF-1 element (-59 to -54) is flanked by a CRE site (-82 to -75) upstream of the TCF-1 element and two sites downstream; the SIE (-43 to -38) and the γ IRE (-37 to -30).

Response elements for the cyclic AMP response element binding protein/activation transcription factor or CREB/ATF family of proteins have been implicated in the regulation of other HCMV early promoters (23, 89, 152). Activation of the UL54 promoter relies on an ATF-1 binding site for the regulation of the polymerase gene. UL112-113 (2.2 kb RNA) promoter activation depends upon a CREB binding site as well. The regulatory region of the US11 early gene contains a CREB and an ATF site, both of which are important in the activation of US11 gene expression. Although UL54, UL112-113, US11, and UL98 are all classified as early genes, each varies in its expression pattern. The genes for the UL54 and the UL112-113 are of the subclass which maintains a constant amount of expression throughout the course of infection. The UL98 gene expression increases from early to late times, while US11 gene expression is downregulated late in infection. CRE/ATF sites, therefore, may be used differently in the regulation of gene expression. Deletion of the UL98 promoter to -64 (p64CAT), which removes the CRE, did not significantly impact promoter activation, but only reduced activity of the UL98 promoter slightly at each timepoint in superinfection assays (Figure

11). Activation levels were at or above wildtype levels in transient assays (Figure 10). However, mutation of the CRE site resulted in a drop in promoter activity. These data showed that the CRE site in the UL98 promoter may also play a role in promoter activation but may require the assistance of other response elements within the promoter.

Sequences downstream of the TCF-1 element, between -51 and -28 (TATA box) include two additional elements: the sis/PDGF inducible element (SIE) at -43 and the gamma interferon response element (γ IRE) at -37. In the absence of upstream sequences, as observed in the -51 promoter, a repressed phenotype is evident in transient assays. Further deletion to -34 or -28 at the minimal TATA box resulted in the return of promoter activation. It was therefore necessary to determine if repression, observed with the -51 promoter, stems from the loss of TCF-1 element or the presence of an isolated SIE/ γ IRE. In response to HCMV, the -51 promoter activity remained significantly diminished. Promoters deleted to -34 and -28 were also significantly reduced, unlike in transient assays. It was noted however that in both cases (transient and superinfection) the -34 and -28 promoter activity was above -51 promoter activity. The return of promoter activation could be the result of a repressor protein (s) binding to the SIE and/or γ IRE in the absence of the upstream sequences. On the other hand, a multiprotein complex that binds upstream sequences to -64 may be responsible for activation, becoming unstable due to the absence of sequences upstream. Further deletion to -51 then allows for sequences downstream of the TATA box to become dominant and play a role in the activation of the promoters deleted to -34 and -28. An ISRE (interferon stimulated response element) site (GGGAAAGGCAACAG) from +81 to +95 has similarities to the consensus ISRE (GGGAAACCGAAACTGAA) and may play a role.

The 5' regulatory sequences beyond the -28 TATA box, however, were not addressed in these studies.

Analysis by Substitution Mutation (CRE and TCF-1)

To this point, it appears by deletion experiments that sequences between -64 and -51 facilitate the transactivation process, possibly via DNA bending through the putative TCF-1 element. As previously mentioned, some early promoters of HCMV as well as of other herpesviruses (13, 43, 173) utilize a CREB/ATF site in the regulation of early gene expression. For this reason, the HCMV UL98 promoter was next mutated at the TCF-1 site or the CREB site or both mutated simultaneously in the context of the full length promoter (-309 to +112). Studies in which all promoter elements remain full length (from -309) as wildtype except where specifically mutated may provide a more accurate assessment of promoter activation. The next series of experiments involve transfection of wildtype or mutant promoter constructs followed by infection with HCMV. Results clearly showed that the mutation of the CRE site alone or in conjunction with the TCF-1 site diminished UL98 promoter activity by greater than 60% (Figure 15). The promoter mutated at the TCF-1 site was only slightly reduced; an unanticipated result considering the removal of this element by deletion nearly abrogated UL98 promoter activity. In this case, CRE appears necessary while the TCF-1 element may have an accessory role. This falls in line with the role of CRE in transactivation and TCF-1 element in facilitating transactivation. Maintaining this line of thinking, recall that in previous studies in T-cells, a TCF-1 element was necessary to bring flanking sites bound by transactivators into close proximity for the activation of the TCR α chain gene promoter (20, 49, 206).

The cyclic AMP response element binding (CREB) protein, which binds the CRE site, is a member of the b-zip family of proteins that binds DNA as a homodimer or heterodimer (CREB1,2 or ATF1,2,3,4, or CREM). A variety of b-zip proteins are known to function in DNA bending as well (139, 149) and different protein dimers induce distinct DNA bends (87). Jun and Fos members bend DNA toward the major groove while ATF-2 dimers bend DNA toward the minor groove. CREB and ATF-1, however, which favor binding to the CRE site over an AP-1 site, do not induce significant DNA bending. This suggests that some b-zip proteins do not induce directed DNA bending. The CRE site alone bends intrinsically toward the major groove, and interaction with a CREB/ATF protein result in the overall “straightening” of the DNA (121, 139). This is possibly the case within the UL98 promoter. Because the CRE may be straightened by the binding of a CREB/ATF family members (CREB1-2, ATF1-4, or CREM), it is suspected that the promoter relies on the presence of the TCF-1 site to bend the DNA in order for other elements bound by proteins to associate and result in optimal promoter activation.

As previously mentioned, the cellular protein CREB and the viral regulatory protein IE86 interact and function in early promoter activation (163, 180). Although the ability of IE86 to bind to the UL98 sequences was not tested, the supershift studies described above could have been extended to determine if IE86 protein was present in the complex bound to the UL98 CRE sequence. Incubation of HCMV infected cell nuclear extracts with the full length UL98 promoter fragment or the UL98 CRE sequence followed by the addition of antibody directed against IE86 might result in a supershift suggesting the presence of IE86 protein (10).

Do cellular and viral proteins cooperate in the activation of the UL98 promoter?

In the gel shift analyses, mock infected cell nuclear extracts incubated with each fragment or ds oligonucleotide used in gel mobility shift analysis resulted in the formation of some protein/DNA complexes visualized as bands (see figure 21 and 22). Upon virus infection, HCMV infected cell nuclear extracts formed bands additional to those observed in the absence of virus. Those complexes formed in the absence of virus are likely the result of cellular transcription factors binding sites specific for them. Virus infection seems to upregulate cellular proteins and supply viral transcription factors. Several protein/DNA complexes are observed in this case. In the presence of viral or virally induced regulatory proteins, activation of the UL98 early promoter is observed. It is the complex cooperation of both that provides the activation of the promoter.

In figures 24 and 25, although both gel mobility shift assays utilize the same ^{32}P 36 bp radiolabeled probe as well as the same HCMV infected nuclear extracts isolated at 72 hours post infection, the protein/DNA interactions observed are quite different. The differences in binding can be explained by variables other than probe and nuclear extracts used. The gel in figure 24 was run in 1X TBE and the gel in figure 25 was run in one-half X TBE. The use of higher ionic strength buffer systems can enhance the resolution of protein-DNA complexes. This would explain the better resolution of figure 24 over figure 25. The increase in binding complexes observed in figure 24 may have resulted from a second variable: the use of nonspecific carrier DNA. While a poly (dI-dC)- poly (dI-dC) alternating copolymer was used at 5 ul per reaction in figure 24, only 2ul was used per reaction in figure 25. In higher ionic strength, a smaller amount of bulk carrier DNA is required to abolish nonspecific binding of proteins to the probe. However, figure

24 contains both a high ionic strength buffer as well as an increased amount of carrier DNA. Therefore, an increase in binding is evident.

CREB was identified in gel mobility shift assays as a protein that binds CRE in the UL98 promoter. To identify the protein that binds specifically, a CRE specific oligonucleotide probe of 45 bp was generated to test in gel mobility shift assays. Through a series of gel shift assays utilizing 72 hour HCMV infected cell nuclear extracts or CREB protein, binding at the CRE site at -82 was determined to be the protein CREB (Figures 26 and 27). Because antibody supershift binding studies showed that neither ATF-1 or ATF-2 bound to the CRE site utilizing anti-ATF-1 or anti-ATF-2 (Figure 26), CREB likely binds the CRE site as a homodimer. Heterodimeric formation of CREB with another CREB/ ATF family member or with the AP-1 (JUN or FOS) transcription factor family remains a possibility. It has been suggested that the ATF/CREB family members could be activated through phosphorylation via the ERK signaling pathway following HCMV infection (152). Increased phosphorylation through this signal transduction pathway appears to compromise phosphatase activity following virus infection, which in turn means sustained transcription factor activity via ERKs. Therefore, viral gene expression is extended and a productive viral infection is maintained (152).

The ATF-1 antibody failed to supershift as did the CREB antibody. However, its altered complex formation of retarded bands does not rule out the possible presence of ATF-1 protein or the supershifting by ATF-1 antibody. The protein/DNA complex was formed prior to the addition of antibody in figure 26 (lane 2). Not visualizing a supershift in lane 8 led to the conclusion that ATF-1 does not bind. Because of the inability to

resolve the binding complexes more precisely in this gel, the ATF-1 antibody may supershift but is not definitively discernible. To determine if ATF-1 is a participant in binding to the UL98 promoter, an alternative test to determine if the protein binding the UL98 promoter is ATF-1 is to block the complex formation observed in lane 2. The addition of antibody prior to incubation of the probe, thus binding of the ATF-1 antibody to the protein (possibly ATF-1), would prevent the formation of the complex in lane 2. ATF-1 antibody might also be cross-reacting with other ATF//CREB family members (CREB, CREM, ATF-2: see “antibodies” in Materials and Methods section) thereby binding the CREB protein and again blocking complex formation.

It appears that CREB may also bind the putative TCF-1 element in the UL98 promoter. In competition assays involving the TCF-1 element and 72 hours HCMV infected cell nuclear extracts, specific binding could be competed off not only by cold TCF-1 specific excess competitor (figure 27), but also by cold CRE excess competitor. This suggests that CREB and the protein(s) which bind the putative TCF-1 element may interact. The implication that UL98 promoter activation requires DNA bending which is instrumental in increasing protein-protein interaction may mean that proteins other than CREB and the proposed TCF-1 are interacting. Therefore, the interaction between CREB and putative TCF-1 may be indirect and require the participation of a protein(s) capable of binding each. An important factor that must be stated is the fact that the TCF-1 has not been identified in fibroblast cells, but TCF-1 has been exclusively detected in T-cell lineages only (202). HCMV has not been shown to activate TCF-1. However, because a number of factors (SRY, UBF, and mammalian testis-determining factor) have been identified (49, 202) that share the HMG box functional in DNA binding, perhaps a

fibroblast specific protein with activity similar to TCF-1 could be involved in activating the UL98 protein in fibroblasts.

To better understand the cooperation between CRE and TCF-1 element in UL98 promoter regulation, additional experiments that involve mutated elements were carried out, this time in the context of truncated promoters. Previous studies showed that in the presence of viral regulatory proteins the promoters deleted to -85 and -64 were activated to wildtype promoter levels. The question arose as to why a promoter lacking CRE (p-64CAT) functions similar to a promoter with CRE present (p-85CAT), if CRE is in fact necessary for UL98 promoter activation (see figure 10; results section). To answer this question, the same CRE and/or TCF-1 element were mutated in the context of the -85 promoter containing CRE or in the context of the -64 promoter in the absence of CRE. The promoter activity from the construct mutated at CRE in the -85 promoter provided similar results as when mutated within the full length construct. The -85 promoter mutated at CRE was significantly reduced at 24, 48, and 72 hours. The promoter mutated at TCF-1 element was reduced at 48 and 72 hours but was comparable to the p-85CAT control at early times. The differences between the effects of mutations in the context of the wildtype promoter and in the context of the -85 promoter indicates that the upstream elements previously mentioned (SRE and E2F) may play a role in the activation of the UL98 promoter with reference to the TCF-1 element but not CRE. Under these conditions, CRE and TCF-1 element remain significant players in the activation of the UL98 promoter.

In the context of the -64 promoter, there is a gradual decrease in promoter activity as each element 5'-3' is mutated toward the TATA box. The promoter activity observed

with the promoter mutated at TCF-1 element in the context of the -64 promoter (p64TmCAT) was not expected. This promoter not only lacks the CRE site (-82), but also contains a nonfunctional TCF-1 site. The inactivation of the two primary elements would likely result in a significant decrease in promoter activity. However, the activation of the p64TmCAT construct is low at early times, but is well above the p64CAT control construct at late times. Previously, it was mentioned that the TCF-1 element is present on the negative strand. There is currently no direct evidence that the element is functional in the opposite orientation. van de Wetering et. al. assessed the binding specificity of a TCF-1 element in the CD3- ϵ enhancer by methylation interference footprinting utilizing the positive and negative strand DNA of the TCF-1 element (205). The A and G interactions were monitored as an indication of nucleotides in contact with the TCF-1 protein. While contacts were made with the A and G of the positive strand, no contacts were observed with A and G on the negative strand (205). Another possible explanation is that the mutation generated in the TCF-1 site (TCTGTTGG to GAATTCGG) is not sufficient to disrupt TCF-1 binding. In this case, additional mutations in the TCF-1 element could have addressed this issue. Gel mobility shift analysis using the UL98 promoter specific TCF-1 element probe, however, showed specific binding with HCMV specific protein(s) from infected cell nuclear extracts (Figure 28). The TCF-1 element could compete off the specific binding while the mutated TCF-1 element could not. This data suggests that the mutation generated is indeed able to disrupt binding. TCF-1 proteins have been shown to bind specifically to a pyrimidine-rich DNA sequence, 5'-CTTTG-3' (202, 205, 206, 211, 213). The TCF-1 \square (LEF-1) was originally identified as a family of 53-55 kD DNA-binding proteins that bind specifically to a motif (5'-

CCTTTGAA-3') which was present in the TCR α enhancer and the HIV enhancer (20, 213). Although the sequence is no longer pyrimidine rich, a 5'-CTT-3' still exists in the core of the element after replacement with an *EcoRI* site. These data showed that the UL98 promoter is likely dependent upon other transcription factor binding sites as well as CRE and TCF-1-like element.

Analysis by Substitution Mutation (SIE and γ IRE)

Downstream of the TCF-1 binding site were two additional sites: the sis-inducible element (SIE) and the gamma interferon response element (γ IRE). Each was substituted with *EcoRI* sites or both mutated in a single plasmid and transfected into cells followed by HCMV infection. When analyzed at 24, 48, and 72 hours post-infection for promoter activity as compared to wildtype, mutation of the γ IRE and the double mutation of SIE and γ IRE within the promoter resulted in activation 70% below the wildtype promoter remaining decreased throughout the course of infection (Figure 16). While the SIE mutation in the promoter was decreased slightly at 24 and 48 hours, the promoter activity appears to recover to wildtype levels by 72 hours. These superinfection assays showed that the γ IRE and perhaps with the assistance of the SIE also participate in UL98 promoter activation. Mutations of the SIE and/or γ IRE in the context of the -85 promoter provided similar results to the identical mutations in the context of the wildtype promoter (Figure 18).

The sis inducible element (SIE) has been best characterized in the c-fos promoter (60, 158, 159). It is defined by its ability to bind the sis inducible factor (SIF), a PDGF or EGF inducible transcription factor. Studies have shown that while the serum response

element (SRE) is the prominent sequence in c-fos promoter transcription in vitro, the SIE contributes in vivo to c-fos promoter transcription (159). The c-fos promoter is activated independent of the SIE when serum induced, and PDGF and EGF latently induce the promoter in certain cell types (60, 158). These studies on the SIE reported that agents (growth hormones) activate independent responder elements or a set of elements, and so the implication is that multiple independent enhancer elements control c-fos transcription.

The gamma interferon response element (γ IRE) is a member of a family of enhancers that determine the induction of many genes by interferons (IFNs) (reviewed in reference 35). Many variations on a theme are represented here. The element and similar sequences have been referred to as the gamma interferon activation site (GAS) (134), gamma interferon response element (γ IRE) (45, 107, 161, 197), and the gamma interferon response region (GRR) (34). Interferons function to inhibit virus growth and are represented by two types: Type I interferon (IFN α,β) and Type II interferon (IFN γ) (reviewed in reference 207). Type I and II interferons induce the activation of a family of transcription factors called the signal transducers and activators of transcription (STATs). There are six known STATs to date and upon activation form dimers or heterooligomers depending on induction initiated by IFN α,β or IFN γ (160). STATs which are activated in response to IFN α,β form a complex of interferon stimulated gene factors (ISGF3) which consist of STATs 1-3 (91/84 kD, 113 kD, and 48 kD) (161). The ISGF3 complex enters the nucleus to bind the interferon stimulated response element (ISRE) in the promoter/ enhancer region of an activated gene. IFN γ utilizes the same STAT pathway, but can also rapidly induce a second STAT complex called the gamma interferon activation factor (GAF). In this instance, GAF consists of a STAT 1 homodimer (91-kD

STAT protein) that binds the gamma interferon activation sequence (GAS) (34). It should be noted that the SIE is considered a GAS type element and the SIF shares important attributes with STATs.

More recently a family of proteins called the interferon regulatory factors (IRFs) have been described and are candidates for virus-induced ISRE activation (228). There are at least nine IRF family genes in human and mouse. IRF-1 and -2 were identified as transcriptional activator and repressor, respectively (59, 124). IRF-3 was identified by primary sequence homology to the family and shown to be expressed ubiquitously in human tissues (8). Expansion of this group of interferon responsive proteins now includes ISGF3 γ /p48, ICSBP, PIP/ICSAT/IRF-4, IRF-5, IRF-6, and IRF-7 (8, 59, 124). Interferon regulatory factors have also been implicated in early promoter regulation. IRF-3 has the potential to be activated directly by virus infection (131). Additional studies provide strong evidence that IRF-3 and IRF-7 proteins are required for the activation of virus inducible genes (214). IRF-3 and IRF-7 are present in a complex in uninfected cells. In this state, they can neither bind DNA nor activate transcription. Virus infection leads to an increase in the phosphorylation of IRF-3 and possibly IRF-7. This posttranslational modification induces cytoplasm to nucleus translocation where the IRF-3 and IRF-7 proteins are able to bind the promoters of virus inducible genes. The association of IRF-3 with the coactivator p300/CBP following nuclear translocation allows for binding to DNA and participate in the activation of IFN and IFN-stimulated genes (8, 228, 131). This may, in turn, play a role in the HCMV replication. As previously mentioned, γ -IFN has been described as having antiviral effects, but has also been shown recently to positively influence virus replication as well (see Pathogenesis of

HCMV disease in Introduction). ISG's are activated without the need for prior de novo protein synthesis. Protein complexes preexist in a latent form in cell and are activated by phosphorylation and initiate transcription of ISG'S for antiviral defense. On the other hand, ISG's can induce gamma IFN thereby causing the differentiation of monocytes to macrophages which increases the cells susceptibility to virus infection.

A synthetic oligonucleotide of the sequences containing the γ IRE and the SIE were generated and used as a probe to determine if protein(s) from HCMV infected cell nuclear extracts specifically bind this sequence. Because the CRE and TCF-1 element were well isolated by flanking sequences on both sides, it was feasible to generate an oligonucleotide of adequate size or length that only contained the element under study for use as a probe in gel mobility shift analysis. The SIE and γ IRE are six and eight bp in length, respectively and are in tandem with the TATA box. In order to test these sequences in binding assays, the approach was to assess protein-DNA interaction using a 30 bp oligonucleotide which consisted of both transcription factor binding sites along with the TATA box. In figure 29, specific binding to the SIE/ γ IRE probe was observed when using HCMV infected cell nuclear extracts preincubated with excess unlabeled specific 30 bp probe containing SIE and γ IRE. The specificity of SIE/ γ IRE binding (see figure 30) was further determined by testing the binding requirement. Did binding preferentially depend upon one site over the other, or were both sites necessary for the specific interactions observed thus far? In figure 30, competitor oligonucleotides mutated at SIE or γ IRE each competed off specific complexes. Interestingly, the presence of a mutated SIE and an intact γ IRE provided the most efficient competition. This data placed an emphasis on the importance of γ IRE in protein-DNA interaction and possibly

in UL98 promoter activation. UL98 promoter activation experiments utilizing site directed mutagenesis to determine important *cis*-acting sequences revealed that although SIE and/or γ IRE mutations decreased UL98 promoter activity, the γ IRE mutation significantly impacted activity throughout the course of infection. Therefore, γ IRE binding supports a role for γ IRE observed in transfection experiments.

Lastly, an attempt was made to identify the protein(s) that bind with specificity to the SIE/ γ IRE probe. Previous studies demonstrated that when the SIE (AGTTCCCGTCAAT) of the c-fos promoter was tested for binding in gel shift assays, three distinct SIF complexes were identified (159). Three SIF complexes (A-C) were formed and determined to consist of a STAT 3 homodimer (A), a STAT 1,3 heterodimer (B), and a STAT 1 homodimer (C). GAS selected for optimal binding in vitro of STAT 1,3, and 4 have dyad symmetries that are extended towards the center (TTCN₂₋₄GAA) (35). The UL98 promoter SIE/ γ IRE sequences do not resemble the dyad symmetry motif. GAS may also have an extension of the palindrome away from the center of symmetry. A well documented example of this extension of palindrome is the m67 SIE mutant oligonucleotide (also called a high affinity SIE) which converts a weak STAT 1,3 binding site to a strong one (159). On the other hand, the GAS in the two mig (monokine of interferon gamma) chemokine gene promoter contains a weak binding site for STAT 1 (TTANNNTAA) (35). Evidence exists for weak sites in tandem to generate a strong site. Since neither the SIE nor the γ IRE is a consensus binding site, experiments were approached from the standpoint that the two sites likely cooperate to generate a strong (er) STAT binding site.

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation, and immune activation (228). Some IRFs are linked to IFN signal transduction, while others are induced or activated by IFN, and still others are involved in IFN gene regulation. Navarro et al. have shown that HCMV infection results in IRF-3 complexing with the transcriptional coactivator CREB binding protein (CBP) and stimulates transcriptional activation through an ISRE controlled by the ISG54 gene (131).

An IRF-3 antibody was used in supershift assays following the incubation of the 30 bp SIE/ γ IRE probe with 72 hour HCMV infected cell nuclear extracts (Figure 33). Increasing concentrations of anti-IRF-3 antibody supershifted one band consistently suggesting that IRF-3 may be a component of the transacting factors binding the UL98 promoter. To confirm this specificity, the anti-IRF-3 antibody supershift was compared to rabbit antiserum prebleed (Figure 34). Unfortunately, a supershift still occurred in the presence of a nonspecific antiserum. Additional experiments are necessary to confirm this data, perhaps by the use of IRF-3 protein with the SIE/ γ IRE probe and the use of IRF-3 antibody that has since become commercially available (Santa Cruz Biotechnology, Inc.).

IRF-3 is constitutively expressed in a variety of tissues and has a unique feature in response to virus infection. IRF-3 remains in a closed conformation in the cytoplasm of uninfected cells. However, following virus infection or dsRNA, IRF-3 is phosphorylated and a conformational change permits the translocation of IRF-3 into the nucleus. The phosphorylated IRF-3 then enters the nucleus accompanied by the coactivator CBP.

Because of the protein-protein interaction that occurs between the IRF-3 and the coactivator CBP, an anti-CBP antibody could supershift the SIE/ γ IRE probe bound by a phosphorylated IRF-3.

Thus far, site specific mutagenesis has allowed for the identification of three key sequences important for the activation of the UL98 promoter: the cyclic AMP response element, the putative T-cell specific factor element, and the gamma interferon response element. Taken together, mutagenesis studies indicated that the UL98 promoter was activated primarily by CRE and γ IRE although clearly the unique arrangement of all four transcription factor binding sites upstream of the TATA box collectively contribute to the regulation of the UL98 promoter. Each was reduced significantly in infection experiments at particular time points possibly where they have the greatest impact.

All of the observation discussed above relate to the central objective of this project: that is, to characterize the HCMV UL98 promoter by the assessment of the cis-acting elements and transacting factors utilizing extensive site directed mutagenesis and CAT-reporter constructs. The UL98 promoter region as well as promoters containing mutations which unveiled the importance of the CREB binding site will be analyzed in the context of the viral genome as a future continuation of these studies.

Significance of These Studies and Future Directions

The goal of this project was to identify the mechanisms essential for regulating an early gene in HCMV infected cells. In the studies described above, we attempted to identify viral and cellular proteins important for UL98 promoter function as well as promoter sequences through which these proteins act. Transient expression assays were

used to determine viral promoter activity. These studies required the use of the reporter gene chloramphenicol acetyltransferase under the control of the UL98 early viral promoter. The promoter-reporter construct was cotransfected with expression plasmids encoding HCMV IE transcriptional regulatory proteins or cotransfected followed by infection with HCMV.

In results from transient transfection experiments in the presence of HCMV MIE proteins or in the presence of virus on the regulation of the UL98 promoter, two elements play an important role in activation. Our studies, thus far, have identified the cyclic AMP response element (CRE) as the primary regulatory element because it enhances promoter activation at 24, 48, and 72 hours. UL98 promoter activation is decreased by half in the presence of a mutated CRE at all time points. By assessing protein/DNA interaction, it was determined that the cellular factor CREB binds to CRE site in the UL98 promoter. The γ IRE also plays a role however relies upon the presence of the upstream promoter sequences in the regulation of the UL98 early gene. Sequences from -64 to -51 may facilitate the complex interplay between viral and cellular proteins. While CRE appears important throughout the course of infection possibly exerting its regulatory function at both early and late times, γ IRE seems to impact the UL98 promoter late in infection.

By comparison, the UL98 early promoter characteristics in the regulation of early gene expression is markedly similar to other early promoters. Activation occurs via multiple cis-acting elements and both viral and cellular trans-acting proteins; namely IE72, IE86, CREB/ATF, AP-1. CREB/ATF factors in particular seem to play a crucial role in IE and early viral gene expression including promoters of the MIE region, UL54, UL112-113, US11, and UL98. These studies support CREB/ATF family members as

participants in the regulation of viral genes which are germane to the replication of HCMV.

Early gene expression is considered the point of commitment for viral DNA replication. While a number of essential early genes have been characterized thus far, and clearly contribute to the propagation of HCMV, the UL98 gene is nonessential for HCMV replication in tissue culture. The UL98 gene product has been shown to be important in viral egress and therefore aids in the packaging and release of virus (47, 167, 169). It is, therefore, of interest to us to determine if mechanisms which apply to activation of promoters for essential genes reflect general mechanisms of early gene activation or if they are specific for a particular subclass. The UL98 gene is defined as an E3 early subclass gene that is activated early and mRNA increases steadily though late times. The UL98 gene product then functions in maturation. It is necessary to address how the 5' regulatory sequences functionally compares and contrasts in relation to promoters for genes of the E1, E2, and E3 subclasses. Clearly, early viral genes utilize some of the same cellular factors for regulation but also require diverse regulatory mechanisms for their regulated expression from the initiation of transcription to protein stability. These studies may identify key factors involved in activating these genes which may be exploited as targets for the development of effective anti-HCMV therapies.

While transient assays are useful for identifying sequences important for gene expression, this artificial system is limited in displaying the multiple regulatory influences put forth by HCMV during normal viral infection. Additional IE proteins (IRS1/ TRS1, UL36-38) as well as early proteins (UL112-113) of HCMV influence the promoter activation of several early genes. Experiments in which the reporter plasmid

containing the promoter of interest is transfected into cells followed by infection with virus (superinfection) clearly show that additional regulatory proteins are necessary for full promoter activation. In this way, viral transactivators not present in a cotransfection with IE expressing plasmids, can act upon the promoter. It has been shown in previous studies that template-specific differences exist between plasmid DNA and viral DNA (88, 180, 209). Late induction of the 1.2 kb RNA promoter observed in infected cells does not occur when promoter is in a plasmid in transient transfection assays unless origin of replication, *oriLyf*, is present (209). Although one can study gene expression in infected cells, a true reflection of the regulatory events of early gene expression is not evident. In short, the assays used to this point still lack a biologically relevant environment.

Several studies have been undertaken to assess the role of elements in regulating HCMV promoters in the context of the viral genome. Rodems et. al. sought to determine the various response elements within the UL112-113 promoter at different times postinfection (152). In transient assays, the ATF/CREB site is essential to promoter activity and is shown by a mutant ATF/CREB abolishing early RNA synthesis. The transcription at late times is similar to wildtype. A weak IE86 binding site is nonessential, but enhances the transcription of UL112-113 (152). A mutant IE86 is decreased in early expression and is abolished late. Upon the generation of ATF/CREB and IE86 mutants inserted into the viral genome, ATF/CREB functions only at early times and IE86 is required for the activation of late transcription (152). Studies by Kerry et. al. show inverted repeat 1 (IR1) dependent activation of UL54 promoter in transient assays (90). IR1 mutated in the context of the viral genome reflects IR1 independent activation and instead relies upon the transcription factor binding site ATF for activation

at late times (88, 89). These studies reveal a more precise view of the regulatory events that take place in HCMV early promoter activation and is observed when the promoter sequences are inserted into and tested in the viral genome.

To more precisely evaluate the multiple regulatory influences of the virus on UL98 promoter activation, the wildtype and relevant mutant promoters will be studied in the context of the HCMV genome in the established recombination system utilized by Rodems and Kerry. In the following studies, the promoter-CAT constructs previously generated for mutagenesis studies will be inserted into the viral genome in place of the β -glucuronidase gene located in a nonessential region of the RV134 strain of the HCMV genome. More specifically, mutations that may affect response elements in transient analysis will be cloned into the recombination vector pRC to target insertion into the US9-10 intergenic region of the HCMV genome. The constructs are then transfected into HFF1 cells in the presence of RV134 DNA. RV134, which expresses the marker β -glucuronidase, will lose the marker gene after recombination and the phenotype of the progeny virus will change from blue to white plaques. The viruses positive for a white plaque phenotype will then be isolated by plaque purification methods and characterized for the appropriate insertion of the promoter-CAT construct and proper growth kinetics compared to wildtype RV134. The viruses will then be tested for levels of CAT RNA by northern blot analysis and protein by western blot analysis during infection.

CHAPTER VI

CONCLUSIONS

The HCMV UL98 early promoter of the alkaline exonuclease gene was characterized to better understand the events that occur during the early phase of virus replication. The specific sequences involved in conferring UL98 promoter activation by immediate early (IE) proteins as well as the identification of viral and cellular proteins in the regulation of early gene expression was investigated. To determine the sequences important for the activation of the UL98 promoter, the UL98 regulatory sequences were tested in transient expression assays in a reporter-CAT construct. The promoter was activated synergistically by IE proteins IE72 and IE86. Deletion analysis revealed that a 13 bp sequence from -64 to -51 was essential for activation by viral regulatory proteins. The specificity of sequences necessary for UL98 promoter activation was also determined. Specific cellular response elements within and flanking the 13 bp region were mutated by site directed mutagenesis. Two elements, the cyclic AMP response element (CRE) and the gamma interferon response element (γ IRE), when mutated in the context of the UL98 promoter, negatively impacted promoter function in response to HCMV infection throughout the course of infection. Cellular factors were found to bind UL98 promoter sequences. As a result of binding assays utilizing specific cellular response elements in the presence of HCMV infected cell nuclear extracts, the cellular transcription factor CREB was found to be a component of the proteins that bind the UL98 promoter region. Specific binding was observed for interferon regulatory elements as well, although the protein(s) involved were not identified in these studies. In

summary, the overall conclusion in the characterization of the regulatory sequences of the UL98 early gene was that regulation of early gene expression requires the cooperation of the specific promoter sequences CRE and γ IRE. The HCMV viral proteins IE72 and IE86 in addition to the cellular protein CREB facilitate this activation through protein/DNA binding and likely protein-protein interactions. The assessment of the UL98 promoter sequences is consistent with regulation of other HCMV early promoters. Future studies in the context of the viral genome may provide a more accurate view of the multiple regulatory influences in UL98 gene expression.

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APPENDIX I

LIST AND DESCRIPTION OF PLASMID DNAs OBTAINED FROM OTHER SOURCES OR PREVIOUSLY CONSTRUCTED.

- pSVOCATd-** contains the chloramphenicol acetyltransferase gene and is derived from pGEM vector.
- pSVOd-** vector used to construct IE gene region plasmid.
- pSVCC3-** plasmid containing the IE1 gene region plasmid.
- pACC-** same as pSVCC3 except contains the IE2 gene region plasmid.
- pIE72kD-** IE1 cDNA under control of MIEP, codes for the 72 kD protein.
- pIE86kD-** same pIE72kD except IE2 cDNA codes for the 86 kD protein.
- pSVH-** IE1/IE2 gene region plasmid.
- pCBX8.1-** 8.1 kb *Bam*HI to *Xba*I fragment of *Xba*I-C containing the pp28 gene region.
- ppolCAT-** contains the HCMV polymerase promoter (-460 to +20) cloned into pSVOCATd.

APPENDIX II.

LIST OF PLASMIDS CONSTRUCTED DURING THIS STUDY.

pUL98CAT- UL98 promoter fragment (-309 to +112) of 421 base pairs (bp) cloned into reporter CAT construct pSVOCATd.

pdH309CAT- same plasmid as pUL98CAT except the downstream *Hind*III site at +112 has been inactivated.

Deletion Mutants: p*CAT- where * indicates site of specific deletion or mutation.

p-194CAT- pdH309CAT deleted from *Hind*III (-309) to *Nar*I (-194).

p-136CAT- pdH309CAT deleted from *Hind*III (-309) to *Sun*I (-136).

p-85CAT- pdH309CAT deleted from *Hind*III (-309) to *Hind*III inserted at -85.

p-64CAT- pdH309CAT deleted from *Hind*III (-309) to *Hind*III inserted at -64.

p-51CAT- pdH309CAT deleted from *Hind*III (-309) to *Msc*I (-51).

p-34CAT- pdH309CAT deleted from *Hind*III (-309) to *Hind*III inserted at -34.

p-28CAT- pdH309CAT deleted from *Hind*III (-309) to *Hind*III inserted at -28.

Substitution Mutants (context specific):**Wild-type promoter:** p*CAT- where * indicates site of mutation.pCm5CAT- 5' portion of CRE site replaced with an *EcoRI* site (GAATTC).pTmCAT- TCF replaced with an *EcoRI* site (GAATTC).pCmTmCAT- both CRE and TCF replaced with an *EcoRI* site (GAATTC).pSmCAT- SIE replaced with an *EcoRI* site (GAATTC).pImCAT- γ IRE replaced with an *EcoRI* site (GAATTC).pSmImCAT- both SIE and γ IRE replaced with an *EcoRI* site (GAATTC).**-85 mutants:** p-85*CAT- where * indicates site of mutation.p-85CmCAT- CRE site replaced with an *EcoRI* site.p-85TmCAT- TCF site replaced with an *EcoRI* site.p-85CmTmCAT- both CRE and TCF sites replaced with an *EcoRI* site.p-85SmCAT- SIE site replaced with an *EcoRI* site.p-85ImCAT- γ IRE site replaced with an *EcoRI* site.p-85SmImCAT- both SIE and γ IRE sites replaced with an *EcoRI* site.**-64 mutants:** p-64*CAT- where * indicates the site of mutation.p-64TmCAT- TCF site replaced with an *EcoRI* site.p-64SmCAT- SIE site replaced with an *EcoRI* site.p-64ImCAT- γ IRE site replaced with an *EcoRI* site.p-64SmImCAT- both SIE and γ IRE sites replaced with an *EcoRI* site.

-51 mutants: p-51*CAT-where * indicates the site of mutation.

p-51SmCAT- SIE site replaced with an *EcoRI* site.

p-51ImCAT- γ IRE site replaced with an *EcoRI* site.

APPENDIX III.

LIST OF PRIMERS USED FOR THE GENERATION OF DELETION AND SUBSTITUTION MUTATIONS BY OVERLAPPING PCR MUTAGENESIS.

General primers used in all overlapping PCR mutagenesis reactions and d primers

5'aa- 5'GCATCTGTGCGGTATTTACACCG3'*

5'aaa- 5'CCACCTCTGACTTGAGCGTCGATT3'*

3'dd- 3'GGATATTGGTCTGGCAAGTCGACCS'

(*Note: 5' primers contain different G/C content necessary for match with G/C content of internal primer.)

Primers generated for deletions (c and b primers)

HSI(s)(*Hind*III between SIE and IRE)(-34)-

5'GGCCACGAGCGAAAGCTTGTATTTATATAACGCCTTTCGGCGC3'

HSI(as)(-34)-

3'CCGGTGCTCGCTTTCGAACATAAATATATTGCGGAAAGCCGCG5'

HMT(s)(*Hind*III at the Minimal TATA)(-28)-

5'GCGACGGGCTGAAGCTTTATAACGCCTTTCG3'

HMT(as)(-28)- 3'CGCTGCCCCGACTTCGAAATATTGCGGAAAGC5'

HIC(s)(*Hind*III between Inverted repeat and CRE)(-85)-

5'TCGCAGACCATGAAGCTTGAATACGTCAGAAAGAACGTG3'

HIC(as)(-85)- 3'CAGCGTCTGGTACTTCGAACTTATGCAGTCTTTCTTGCA5'

HCT(s)(*Hind*III between CRE and TCF)(-64)-

5'GAATACGTCAGAAAGAAGCTTGAGCGTCTGTTGGCCACGA3'

HCT(as)(-64)- 3'CTTATGCAGTCTTTCTTCGAACTCTCATACAACCGGTGCT5'

Primers generated for substitution mutations

CRE-1m- 5'CCATGCTGCACGAAGAATTCAGAAAGAACGTGGAG3'

CRE-2m- 3'GGTACGACGTGCTTCTTAAGTCTTTCTTGCACCTC5'

TCF-1m- 5'GAAAGAACGTGGAGCGGAATTCGGCCACGA3'

TCF-2m- 3'CTTTCTTGCACCTCGCCTTAAGCCGGTGC5'

CmTm(s)-

5'GCTGCACGAAGAATTCAGAAAGAACGTGGAGCGGAATTCGGCCAC3'

CmTm(as)-

3'GACGTGCTTCTTAAGTCTTTCTTGCACCTCGCCTTAAGCCGGTGC5'

SIEm2(s)-

5'CCACGAGCGAATTCCTGTATTTATATAACGCCTTTCGGCGCACCAACAGC3'

SIEm2(as)-

3'GGTGCTCGCTTAAGGACATAAATATATTGCGGAAGCCGCGTGGTGGTCG5'

γ IREm(s)- 5'CCACGAGCGACGGGGAATTCTTATATAACGCCTTTCG3'

γ IREm(as)- 3'GGTGCTCGCTGCCCCCTTAAGAATATATTGCGGAAAGC5'

SmIm(s)- 5'GGCCACGAGCGAATTCGAATTCTTATATAA3'

SmIm(as)- 3'CCGGTGCTCGCTTAAGCTTAAGAATATATT5'

VITA

I was born in Groton, Connecticut, USA on February 10, 1968. I obtained my Bachelor's degree from Norfolk State University in 1991. My major was Biology with an emphasis in Premed. As a Sophomore, I received an undergraduate research fellowship in the MARC (Minority Access to Research Careers) Program awarded by the National Institute of General Medical Sciences. I served as a research fellow while a full time student until graduation.

After my graduation, I was awarded a one year research fellowship by the National Cancer Institute in the Comprehensive Minority Biomedical Program. I did basic science research in the area of molecular biology. Upon acceptance into a Ph.D. graduate school program in 1991, I deferred my studies one year at which time I obtained additional experience in a clinical Cytogenetics laboratory. In 1993, began the pursuit of my Doctoral degree in Biomedical Sciences.

Interested in the importance of understanding viral pathology especially concerning HIV, I was afforded the opportunity to study HCMV, a different human pathogen, under the direction of Dr. Richard M. Stenberg (Eastern Virginia Medical School, Lewis Hall, Department of Microbiology and Molecular Cell Biology, 700 W. Olney Road, Norfolk, VA. 23507) via a MARC predoctoral fellowship. Obtaining a significant amount of knowledge in the regulation of HCMV early genes, I presented research data in several local and national meetings earning awards from Eastern Virginia Medical School and the American Society for Microbiology. At present, a manuscript is in preparation for submission for publication.